

Atty. Dkt. No. 028622-0102
Appln. No. 09/673,735

REMARKS

I. Introduction

Applicants respectfully request reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow. In addition, Applicants thank the Examiner for the courtesy of a phone interview regarding this application on February 17, 2005.

Claims 6, 17-19, 24-29, 31-32, 34-36, 38-39, and 42-43 are requested to be cancelled. The cancellation of claims does not constitute acquiescence in the propriety of any rejection set forth by the Examiner. Applicants reserve the right to pursue the subject matter of the canceled claims in subsequent divisional applications.

Claims 1, 21, and 30 are currently being amended.

This amendment adds, changes and/or deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claims remain under examination in the application, is presented, with an appropriate defined status identifier.

Upon entry of this Amendment, claims 1-5, 7-16, 20-23, 30, 33, 37, 40-41, and 44-45 will remain pending in the application.

Because the foregoing amendments do not introduce new matter, entry thereof by the Examiner is respectfully requested.

II. Response to Issues Raised by Examiner in Outstanding Office Action

a. Claim Rejections - 35 U.S.C. § 112, First Paragraph

Claims 11 and 44-45 are rejected by the Examiner under 35 U.S.C. § 112, first paragraph for lack of enablement. Applicants respectfully request reconsideration and withdrawal of the rejection.

The Examiner asserts, “while it is known that some and maybe not all CDRs are needed for contacting antigen, as stated in Rudikoff the CDRs are in the context of structural requirements and even though one may not contact antigen it is still needed in the structural role for the other CDR contacting residues.” See Office Action 9/27/04, p. 3. In addition, the Examiner believes neither the Olsen nor Sompuram papers are representative of immunoglobins in general and are not persuasive to one of skill in the art. Id.

The Examiner argues that a person of skill in the art “would not expect the antibodies as claimed which do not have a full set of CDRs to bind antigen.” Office Action dated 9/27/04 p. 3. Applicants believe that enablement should not be measured by the expectations of one skilled in the art. Rather, “[a]ny analysis of whether a particular claim is supported by the disclosure in an application requires a determination of whether that disclosure, when filed, contained sufficient information regarding the subject matter of the claims as to enable one skilled in the pertinent art to make and use the claimed invention.” MPEP, 8th ed. Rev.2, 2164.01. See also *United States v. Telecommunications, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988) (“The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation.”). A patent need not teach, and preferably omits, what is well known in the art. *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), cert. denied, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).

The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *In re Certain Limited-Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), aff'd. sub nom., *Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985). See also *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404.

As long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the

enablement requirement of 35 U.S.C. 112 is satisfied. *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). A person of skill in the art would be able to make and use CD19 x CD3 single chain constructs with fewer than a full compliment of CDRs and test their specific binding using methods within the specification. Such tests would indicate whether the new antibody still showed specific binding and would, therefore, fall within the scope of the claims.

There are many factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is "undue." These factors include, but are not limited to: 1) The breadth of the claims; 2) The nature of the invention; 3) The state of the prior art; 4) The level of one of ordinary skill; 5) The level of predictability in the art; 6) The amount of direction provided by the inventor; 7) The existence of working examples; and 8) The quantity of experimentation needed to make or use the invention based on the content of the disclosure. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988) (reversing the PTO's determination that claims directed to methods for detection of hepatitis B surface antigens did not satisfy the enablement requirement). In *Wands*, the court noted that there was no disagreement as to the facts, but merely a disagreement as to the interpretation of the data and the conclusion to be made from the facts. *In re Wands*, 858 F.2d at 736-40, 8 USPQ2d at 1403-07. The Court held that the specification was enabling with respect to the claims at issue and found that "there was considerable direction and guidance" in the specification; there was "a high level of skill in the art at the time the application was filed;" and "all of the methods needed to practice the invention were well known." 858 F.2d at 740, 8 USPQ2d at 1406. After considering all the factors related to the enablement issue, the court concluded that "it would not require undue experimentation to obtain antibodies needed to practice the claimed invention." *Id.*, 8 USPQ2d at 1407.

In re Wands is informative for the Applicant's patent due to the similarity in technology and status of the pending examinations. Similar to *Wands*, Applicant's specification involves antibody technology and the claims at issue require the use of known techniques to create, screen, and identify antibodies with certain binding characteristics. In

many ways Applicant's techniques are more predictable than *Wands*. *Wands* first had to create and select hybridomas from which to isolate antibodies and then later perform an additional step of selection based on binding affinity. Applicant enables a one step selection process following the creation of antibodies using standard recombinant technology.

For all of the factors described above, Applicant believes the present application enables a person of ordinary skill in the art at least as well as the application in *Wands* enabled its claims. The breadth of the claims in *Wands* are to an immunoassay comprising a broad antibody that can bind a specific target. Applicant's claims are directed to a CD19 x CD3 single chain construct that recognizes the CD19 and CD3 antigens. Both inventions involve antibody technology and, therefore, constitute a similar nature of the invention. Applicants specification was submitted over 15 years after the *Wands* application, so the state of the prior art and the skill of the ordinary person in the art increased due to 15 years of research and prior art disclosures.

The level of predictability in the Applicant's specification is comparable to *Wands*. Applicants describe known recombinant techniques for producing specific antibodies for testing. *Wands* selects antibodies from numerous hybridomas. Both *Wands* and Applicant require purification of antibodies and further screening to determine if the antibody had the required binding constant.

The amount of direction provided by the Applicant and the existence of working examples are described below. The specification discloses the common use of PCR and cloning technology to create constructs of interest for overexpression and purification. On page 7 references to techniques for performing insertions, deletions, substitutions and mutations is described. Examples 1 and 2 from pages 32-34 of the specification describe in detail the preparation of some of the constructs in the application. Extensive information on the proper use of primers, restriction enzymes, and vectors for overexpression is provided. Methods of overexpression and purification of the desired construct are found on page 34. Additionally, the specification discloses how to determine whether the single-chain multi-functional polypeptide binds to the target antigens, CD19 and CD3. Example 3 beginning on page 34, teaches where such binding is determined by FACS analysis.

The quantity of experimentation required for these experiments is commensurate with normal scientific activity in the field of immunology. For *Wands*, the court allowed considerable experimentation:

[I]n the monoclonal antibody art it appears that an "experiment" is not simply the screening of a single hybridoma, but is rather the entire attempt to make a monoclonal antibody against a particular antigen. This process entails immunizing animals, fusing lymphocytes from the immunized animals with myeloma cells to make hybridomas, cloning the hybridomas, and screening the antibodies produced by the hybridomas for the desired characteristics

Wands, 858 F.2d, at 740. Applicants submit that experiments required to enable these claims are less burdensome than those described above. The specification discloses how a person skilled in the art can test for the specific binding of constructs to the target antigens using standard techniques that do not require undue experimentation. In light of these disclosures, Applicant's respectfully request reconsideration and withdrawal of this rejection.

b. Claim Rejections - 35 U.S.C. § 103

Claims 1-16, 20-23, 30, 33, 35-43, and 44-45 are rejected by the Examiner under 35 U.S.C. § 103(a) as being unpatentable over Bohlen et al (Blood 82: 1803-1812, 1993) ("Bohlen") and further in view of Mack et al (PNAS 92: 7021-7025, 1995) ("Mack") and as evidenced from the specification and Blattler et al. (US Patent 5,239,062) ("Blattler"). Office Action dated 9/27/04, p. 3. Applicants respectfully request reconsideration and withdrawal of the rejection.

The Examiner asserts that Bohlen teaches a bispecific antibody that binds CD19 and human CD3, a method of preparing the antibody, and a method of treatment with the antibody. Office Action dated 1/20/04, p. 8. However the Examiner concedes that Bohlen does not teach a single chain bispecific CD19 x human CD3 antibody or methods to treat non-Hodgkin's lymphoma with an antibody. Id. The Examiner contends that Mack and Blattler make up these deficiencies. The Examiner cites Mack as disclosing a single chain bispecific antibody that binds CD3 and 17-1, methods of treatment with this antibody, and methods of making this single-chain antibody. Id., p. 8-9. The Examiner has applied Blattler as teaching that the CD19 antigen is expressed in all B-CLL and all non Hodgkin's lymphomas. Id., p. 9.

The Examiner concludes that it would have been *prima facie* obvious to one of ordinary skill in the art at the time that the invention was made to produce a single-chain bispecific antibody from the bispecific antibody of Bohlen by the method of Mack for the treatment of non-Hodgkin's lymphoma. Id. The Examiner further alleges that one skilled in the art would have been motivated and had a reasonable expectation of success to have produced a single-chain bispecific antibody as set forth above because Bohlen teaches that a bispecific binding agent which binds CD19 and human CD3 can be used for the treatment of B-CLL and as taught by Blattler, the CD19 antigen is expressed in all B cell non-Hodgkin's lymphomas. Id., p. 9-10. Therefore, the Examiner further alleges that it would have been obvious to treat Non-Hodgkin's lymphoma with a bispecific molecule, a bispecific antibody directed to CD19 and CD3. Id., p. 10.

To establish a *prima facie* case of obviousness, however, there needs to be (1) some suggestion or motivation to modify the reference or to combine reference teachings, (2) a reasonable expectation of success, and (3) the prior art references, when combined, must teach or suggest all the limitations of the claimed invention. See MPEP §2143. "Both the suggestion and the reasonable expectation of success must be founded in the prior art, not in the applicant's disclosure." *In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991). Applicants respectfully assert that the examiner has not met his burden.

In order to expedite prosecution and without prejudice to pursuing original claim 1 in a continuing application, Applicants have amended independent claims 1, 21, and 30 to include the limitation of a single chain multi-functional peptide arranged in the order V_LCD19-V_HCD19-V_HCD3-V_LCD3. Nevertheless, Applicant reasserts arguments made in the previously filed Amendment of July 20, 2004. In addition, Applicant also asserts that Bohlen in fact teaches away from the present claimed invention because Bohlen additionally requires the use of an anti-CD28 co-stimulatory antibody to achieve its cytotoxicity (see abstract and Table 3 of Bohlen). The presently claimed invention does not require any costimulatory agents and in fact teaches on page 4, first full paragraph of the specification that no costimulatory agents are necessary. MPEP §2144.04 II (B) states that the omission of an element and retention of its function is an indicia of unobviousness, citing *In re Edge*, 359

F.2d 896, 149 USPQ 556 (CCPA 1966). This is clearly the case for the presently claimed invention.

Applicants also believe that an analysis of the prior art at the time of filing would not lead a person of ordinary skill in the art to this claimed invention. Kipriyanov published multiple studies on the use of a CD3 x CD19 antibody in the lysis of malignant human B-cells. See Kipriyanov et al, Abstract for the Fourteenth International Conference on Advances in the Application of Monoclonal Antibodies in Clinical Oncology, Greece, May 1997 (“Kipriyanov Abstract”) and Kipriyanov, et al., Bispecific CD3 x CD19 Diabody for T Cell-Mediated Lysis of Malignant Human B Cells, *Int. J. Cancer*, 77, 763-772 (1998) (“Kipriyanov 1998 Article”) (See Attached A and B). Kipriyanov pursued multiple avenues of research including the creation of single chain antibodies. See Kipriyanov Abstract, second paragraph, and Kipriyanov 1998 Article, p. 769. As the Kipriyanov 1998 Article clarifies, the single chain antibodies were constructed as recommended by Gruber. See Kipriyanov 1998 Article, p. 769, referencing Gruber, et al., Efficient Tumor Cell Lysis Mediated by a Bispecific Single Chain Antibody Expressed in Escherichia coli, *J. Immunol.*, 152(11): 5368-74.(1994) (“Gruber”) (See Attached C).

Gruber constructed a bispecific single chain antibody of 1B2 (Anti T-Cell Receptor) x 4420 (Anti-Fluorescein) described as the TCR/fluorescein bispecific Ab with the following structure V_L1B2-V_H1B2-V_L4420-V_H4420. In addition, a 10 amino acid peptide from the c-myc protein was placed at the C-terminus. See Gruber, Figure 1 and p. 5369-70. Following the same procedure as Gruber, the Kipriyanov CD3 x CD19 construct would have the following structure:: V_LCD3-V_HCD3-V_LCD19-V_HCD19. See Kipriyanov 1998 Article, p. 769. Kipriyanov attempted to take advantage of a bispecific CD3 x CD19 single chain antibody and found the antibody to provide no (Kipriyanov Abstract) or low (Kipriyanov 1998 Article, p. 769) recognition of CD3.

Although Gruber successfully uses his bispecific single chain antibody to achieve high levels of binding for both antibody targets, Gruber recognizes the possibility of steric hindrance preventing proper functioning of other Abs in this bispecific single chain context. See Gruber, p. 5373. Following the experiments of Kipriyanov, a person of ordinary skill in

the art would conclude that a bispecific single chain construct of CD3 and CD19 had been tested and could not properly function.

The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, not in applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). "It is difficult but necessary that the decision maker forget what he or she has been taught ... about the claimed invention and cast the mind back to the time the invention was made (often as here many years), to occupy the mind of one skilled in the art who is presented only with the references, and who is normally guided by the then-accepted wisdom in the art." *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540, 220 USPQ 303, 313 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851 (1984). Mack's experiments would only clarify to a person of skill in the art that CD3 has one partner (17-1A) where the CD3 affinity in a single chain antibody is still high. However, as predicted by Gruber, other bispecific antibodies may cause steric hindrance and prevent proper functioning of at least one of the antibody constituents, as seen in Kipriyanov with CD3 and CD19. In light of the experience of Kipriyanov, Mack would not suggest or motivate a person of ordinary skill in the art to combine the reference with Bohlen, nor would there be a reasonable expectation of success in the creation of a CD19 x CD3 construct. Rather one would conclude that Kipriyanov, a study published after Mack, provides sufficient evidence for not further pursuing the single chain CD19 x CD3 construct.

The ability to swap entire domains in the context of immunoglobins and retain proper binding for both domains individually is an unpredictable event. Antibodies have two binding sites to bind receptors on cells or other antigens of interest. Removal of one can prevent proper functioning by lowering the overall specificity of binding. Alternatively, the use of a different domain may affect domain-domain interactions in such a way as to destabilize proper functioning.

Many aspects of protein binding can be affected by slight changes in the three dimensional structure of a protein. Creating a single chain bispecific antibody with separate immunoglobulin domains can have additional unexpected consequences. The linker between the two domains may affect the stability of the compound or restrict the orientation between

the domains and prevent proper functioning of the single chain antibody. All of these factors would cast doubt to one skilled in the art as to whether the formation of a CD19 x CD3 single chain construct would work properly.

Although Mack is successful in creating a functional 17-1A x CD3 antibody, a person of ordinary skill in the art would have no reasonable expectation that a different pair with CD3 would have the same success. After the experience of Kipriyanov, the use of a CD19 x CD3 pair in a single chain format would certainly be questioned and likely lead to the pursuit of a diabody approach that had shown promise in the case of CD19 x CD3. See Kipriyanov 1998 Article. Although single chain experiments may be more routine today, expectation of success must be measured at the time of invention.

In addition to the above, other reasons indicate that a combination of Mack with Bohlen would not be obvious to treat B-Cell diseases and Non-Hodgkin's Lymphoma. The Examiner suggests that treatment of the above diseases provides the motivation to combine Bohlen with Mack and such a combination would have a reasonable expectation of success. See Office Action dated 1/20/04, p. 9. This motivation is necessary because no mention of CD19 appears Mack.

The Mack 17-1A x CD3 construct is directed to the 17-1A epitope, also known as EpCAM, on solid tumors. EpCAM has been shown to be a target for monoclonal antibody therapy in patients with minimal residual colorectal cancer. See Mack, p. 7021. The targeted antigen (17-1A) is present on colorectal cancer cells which form solid tumors in patients. Although the EpCAM (17-1A) antigen is characteristic for solid tumor, colorectal cancer, the CD19 antigen is expressed on lymphatic, hematologic target cells. Mack suggests the use of his 17-1A x CD3 construct for the treatment of "colorectal cancer patients with disseminated residual tumor cells after complete resection of their primary tumor." See Mack, p. 7025. These patients have only a low tumor load with minimal remnants of solid cancer requiring treatment. Patients with B-CLL and Non-Hodgkin's Lymphoma have a high tumor load and no solid tumors available for initial resection.

Mack's techniques do not have a reasonable expectation of success for the treatment of B-CLL and Non-Hodgkin's Lymphomas. A person of ordinary skill in the art may see

Mack as useful in treating small amounts of a solid tumor where the majority of cancer has been resected, but would not see Mack as providing support for the treatment of high tumor load, pervasive cancer throughout the entire hematologic system. There is no motivation for a person skilled in the art to use information from a construct directed against residual cells from resected solid tumors to a construct intended for use in the elimination of cancerous B-cells. The present invention successfully treats patients with high tumor load, without requiring the resection of the primary tumor. As demonstrated in Example 7, administration of the invention to an end-stage B-CLL- (B cell-derived chronic lymphatic leukemia) patient characterized by a high tumor load led to a 20% size reduction of lymph nodes and spleen due to destruction of malignant B cells.

Furthermore, although a person of ordinary skill in the art would recognize that Blattler teaches the presence of CD19 antigens in all B-CLL and all non Hodgkin's lymphomas, the teachings of Kipriyanov, as described above, lead away from the creation of a single chain CD19 x CD3 construct to treat the disease. In addition, our analysis of Mack indicates why use of the present invention to treat B-CLL and Non-Hodgkin's Lymphoma is not obvious from the prior art.

After reading the specification of the Applicant which describes the success of the claimed constructs in treating disease, improper hindsight may lead to the conclusion that this was obvious at the time of filing. However, based on the a reading of the entire state of the prior art and a close look at the limited scope of Mack, a person of ordinary skill in the art would not have deemed the present invention obvious. In addition, the arguments described above teach away from a suggestion to combine references for an obvious rejection. Applicants respectfully request reconsideration and withdrawal of this rejection.

CONCLUSION

The present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

It is acknowledged that the foregoing amendments are submitted after final rejection. However, because the amendments do not introduce new matter or raise new issues, and because the amendments either place the application in condition for allowance or at least in better condition for appeal, entry thereof by the Examiner is respectfully requested.

Applicants believe the application is in condition for allowance. However, in order to maintain pendency of the application, Applicants are filing a Notice of Appeal.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants hereby petition for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date March 28, 2005

By Richard Peet

FOLEY & LARDNER LLP
Customer Number: 22428
Telephone: (202) 672-5483
Facsimile: (202) 672-5399

Richard Peet
Attorney for Applicant
Registration 35,792

S. Kiprithanor

DAD

The Fourteenth International Conference

on

**Advances in the Application of Monoclonal Antibodies
in Clinical Oncology**

5-7 May 1997
Thira, Santorini, Greece



(The Minoan fisherman of Santorini)

Sponsored by:

Antisoma

*Purdue
Pharma L.P.*

BIOMIRA

Bispecific diabody for lysis of human B-lineage leukaemia cells

S.M. Kipriyanov¹, G. Moldenhauer², G. Strauss², M. Little¹

¹Recombinant Antibody Research Group (044S), Diagnostics and Experimental Therapy Programme; ²Dept. of Molecular Immunology (0740), Tumour Immunology Programme, German Cancer Research Centre (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

Recombinant antibody fragments directed against cell surface antigens can provide useful components for the development of therapeutic agents. To target cytotoxic effector T-cells to B-lineage leukaemia cells expressing the CD19 differentiation marker, we have constructed anti-human CD3 and anti-human CD19 single-chain antibodies by PCR amplification of immunoglobulin variable domain genes from cDNAs of the hybridomas OKT3 and HD37, respectively. Cloning the correct genes coding for the antibody variable domains (especially V_L kappa) was complicated by the presence of several immunoglobulin transcripts, some of them arising from the myeloma cell line. For the rapid functional evaluation of recombinant antibody fragments against cell surface antigens, we combined a colony screening assay with the analysis of crude periplasmic extracts by flow cytometry. This procedure facilitated the efficient cloning of a functional V_H/V_L combination from the hybridoma cDNA. As a part of the anti-CD3 scFv construction process, the PCR amplified OKT3 V_H gene was modified to improve its *in vivo* folding. These modifications resulted in a dramatic increase of the yield and stability of soluble scFv. We also found that the addition of the non-metabolised sugar (sucrose) to growth medium after induction appeared to stabilise folding intermediates, leading to a further increase in the amount of soluble and functional scFv.

For the creation of bispecific anti-human CD3-CD19 antibodies, two different strategies were utilised. A single chain (scFv)₂ antibody, where the individual scFv regions were joined by a 20 amino acid linker, and a non-covalent heterodimer diabody were constructed. The soluble diabody proved to be produced by *E.coli* at a much higher yield than (scFv)₂, consisted of only dimers after IMAC purification and specifically interacted with both CD3 and CD19 positive cells. The (scFv)₂ construct, however, failed to recognise human CD3. The diabody binds CD3 and CD19 cell surface antigens with affinities close to those of parental scFvs. It was less stable than anti-CD3 scFv but more stable than anti-CD19 scFv when incubated in human serum at 37°C. The diabody is potent in retargeting peripheral blood lymphocytes to lyse tumour cells expressing the CD19 antigen. The cytotoxic activity of the diabody was increased by preincubation with effector cells and costimulation using a bivalent anti-human CD28 antibody.

1. Kipriyanov SM, Kupriyanova OA, Little M, Moldenhauer G (1996) Rapid detection of recombinant antibody fragments directed against cell-surface antigens by flow cytometry. *J. Immunol. Methods*, 196, 51-62.
2. Kipriyanov SM, Moldenhauer G, Little M (1997) High level production of soluble single chain antibodies in small-scale *Escherichia coli* cultures. *J. Immunol. Methods*, 200, 69-77.
3. Kipriyanov SM, Moldenhauer G, Martin ACR, Kupriyanova OA, Little M (1997) Two amino acid mutations in an anti-human CD3 single chain Fv antibody fragment that affect the yield on bacterial secretion but not the affinity. *Protein Engineering*, in press.

BISPECIFIC CD3 X CD19 DIABODY FOR T CELL-MEDIATED LYSIS OF MALIGNANT HUMAN B CELLS

Sergey M. KIPRIYANOV¹, Gerhard MOLDENHAUER², Gudrun STRAUSS² and Melvyn LITTLE^{1*}

¹Recombinant Antibody Research Group, Diagnostics and Experimental Therapy Program, German Cancer Research Center (DKFZ), Heidelberg, Germany

²Department of Molecular Immunology, Tumor Immunology Program, DKFZ, Heidelberg, Germany

For the treatment of minimal residual disease in patients with leukemias and malignant lymphomas, we constructed a heterodimeric diabody specific for human CD19 on B cells and CD3ε chain of the T cell receptor complex. The bispecific diabody was expressed in *Escherichia coli* using a vector containing a dicistronic operon for co-secretion of V_H-3-V_L19 and V_H19-V_L3 single-chain Fv fragments (scFv). It was purified in one step by immobilized metal affinity chromatography (IMAC) from the periplasmic extract and culture medium. Flow cytometry experiments revealed specific interactions of the diabody with both CD3 and CD19 positive cells, to which it bound with affinities close to those of the parental scFvs. It was less stable than anti-CD3 scFv but more stable than anti-CD19 scFv when incubated in human serum at 37°C. In cytotoxicity tests, the diabody proved to be a potent agent for retargeting peripheral blood lymphocytes to lyse tumor cells expressing the CD19 antigen. The efficiency of cell lysis compared favorably with that obtained with a bispecific antibody (BsAb) of the same dual specificity that was prepared by the quadroma technique. *Int. J. Cancer* 77:763–772, 1998.

© 1998 Wiley-Liss, Inc.

B cell leukemias and malignant lymphomas represent a heterogeneous group of hematological malignancies occurring in blood, lymph nodes and bone marrow, which frequently disseminate throughout the body. The most common forms of non-Hodgkin's lymphoma (NHL) are derived from the B cell lineage. The incidence of NHL (6–17/100,000) continues to increase worldwide at about 4% a year. Although NHL can be treated with reasonable success at early and intermediate stages, the results of conventional chemotherapy and radiation in advanced stages remain disappointing. This holds particularly true for the prevalent low-grade lymphomas. A fairly large number of patients relapse, and most remissions cannot be extended beyond minimal residual disease. In this case, high-dose chemotherapy combined with total body irradiation together with the support of an autologous bone marrow transplantation (BMT) or peripheral blood stem cell transplantation (PBSCT) offers an alternative means of curing the disease. However, an important issue still to be solved concerns the presence of residual tumor cells in the patient that give rise to a recurrence of the leukemia or lymphoma.

To eradicate residual tumor cells, BsAbs have been proposed as a means of recruiting cytotoxic T cells for killing tumor cells (Fanger *et al.*, 1992). For example, clinical studies have shown tumor regression in patients treated with BsAb directed against tumor antigens and the CD3 component of the T cell receptor complex, respectively (Canevari *et al.*, 1995; Nitta *et al.*, 1990). One of the best targets for bispecific antibodies on malignant human B cells is CD19 (Grossbard *et al.*, 1992). This antigen is expressed on virtually all B-lineage malignancies from acute lymphoblastic leukemia (ALL) to NHL. Moreover, it is not shed and is absent from hemopoietic stem cells, plasma cells, T cells and other tissues. A potential disadvantage is that normal B cells may also be killed by CD3 X CD19 BsAb treatment. However, these cells are rapidly replaced by differentiation from the stem cell pool.

Various strategies have been utilized for the creation of BsAbs. Heteroconjugates have been produced by chemical cross-linking of 2 monoclonal antibodies (MAb) (Anderson *et al.*, 1992) or Fab' fragments (Brennan *et al.*, 1985). Alternatively, BsAbs were pro-

duced using hybrid hybridoma (quadroma) technology (Bohlen *et al.*, 1993; Csóka *et al.*, 1996). A major limitation of this procedure is the production of inactive antibodies due to the random L-H and H-H associations. Only about 15% of the antibody produced by the quadroma is of the desired specificity (Milstein and Cuello, 1983). The correct BsAb must then be purified in a costly procedure from a large quantity of other very similar molecules. A further limitation of the quadroma BsAb from rodent cell lines is their immunogenicity. Repeated doses of rodent antibodies elicit an anti-immunoglobulin response, referred to as HAMA (human anti-murine antibody).

Some of the limitations of MAbs as therapeutic agents have recently been addressed by genetic engineering (Winter and Milstein, 1991) including a few methods for BsAb fragment production (Carter *et al.*, 1995). Bispecific F(ab')₂ have been created either by chemical coupling from Fab' fragments expressed in *E. coli* (Shalaby *et al.*, 1992) or by heterodimerization through leucine zippers (Kostelny *et al.*, 1992). Even smaller BsAb fragments have been constructed based on scFv: the association of V_H and V_L domains is stabilized by a flexible polypeptide linker (Bird *et al.*, 1988). The genetic engineering of 2 scFvs linked with a third polypeptide linker, as initially suggested by Huston *et al.* (1991), has now been carried out in several laboratories for the production of bispecific single-chain antibody segments (scFv)₂ with a potential anti-tumor activity (Gruber *et al.*, 1994; Mack *et al.*, 1995).

An alternative BsAb fragment is the scFv heterodimer diabody (Holliger *et al.*, 1993). It is formed by the non-covalent association of 2 single-chain fusion products consisting of the V_H domain from one antibody connected by a short linker to the V_L domain of another antibody (Atwell *et al.*, 1996; Holliger *et al.*, 1996; Zhu *et al.*, 1996). The 2 antigen binding domains have been shown by crystallographic analysis to be on opposite sides of the complex such that they are able to cross-link 2 cells (Perisic *et al.*, 1994).

Starting with the mRNA of hybridoma cells HD37 (Pezzutto *et al.*, 1987) and OKT3 (Kung *et al.*, 1979), we previously constructed recombinant scFv antibody fragments specific for the human B cell antigen CD19 (Kipriyanov *et al.*, 1996) and the CD3ε chain of the human CD3/T cell receptor complex (Kipriyanov *et al.*, 1997b). Here, we describe the construction and production of a bispecific diabody in bacteria with dual specificity for both the human B cell antigen CD19 and CD3/TCR. The diabody has been compared with parental scFvs with respect to its stability in human serum at 37°C and binding affinity to both CD19-positive human B cells and CD3-positive human T cells. In cytotoxic assays, the CD3 X CD19 diabody was able to retarget human PBL to malignant B cells. The efficiency of cell lysis compared favorably with that obtained with a BsAb of the same dual specificity prepared by the quadroma technique.

Grant sponsor: Deutsche Krebshilfe/Mildred Scheel Stiftung.

*Correspondence to: Recombinant Antibody Research Group (D0500), German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany. Fax: (49)6221-423-462.
E-mail: m.little@dkfz-heidelberg.de

Received 23 January 1998; Revised 6 March 1998

MATERIAL AND METHODS

Monoclonal antibodies

The CD3 ϵ -chain-specific hybridoma cell line OKT3 secreting an IgG_{2a} MAb (Kung *et al.*, 1979) was obtained from the ATCC (Rockville, MD). The HD37 cell line produces a MAb (IgG₁) reactive with the human CD19 molecule and has been described in detail (Pezzutto *et al.*, 1987). Monoclonal antibodies were produced in a miniPERM bioreactor (Heraeus, Osterode, Germany) and purified by affinity chromatography on a Protein-A Sepharose CL-4B column (Pharmacia, Uppsala, Sweden). Isolation and characterization of hybrid hybridoma OKT3 \times HD37 has been described (Csóka *et al.*, 1996).

Vector construction

The *E. coli* K12 strain XL1-Blue (Stratagene, La Jolla, CA) was used as the cloning and expression host. Plasmids pHOG- α CD19 and pHOG-dmOKT3 encoding the scFv fragments derived from

hybridoma HD37 specific for human CD19 (Kipriyanov *et al.*, 1996) and OKT3 specific for human CD3 (Kipriyanov *et al.*, 1997b), respectively, were used for assembly of the diabody to create the expression plasmid pKID3 \times 19. Briefly, a PCR fragment of the V_H domain of anti-CD19 preceded by a BglII site and followed by a segment coding for a LysLeuGlyGly linker was generated using the primers DP1, 5'-TCACACAGAAATTCTT AGATCTATTAAAGAGGGAGAAATTAAACC and DP2, 5'-AGCACACGATATCACCGCCAAGCTTGGGTGTTGGC (Fig. 1). The PCR fragment was digested with EcoRI and EcoRV and ligated with the EcoRI/EcoRV linearized plasmid pHOG-dmOKT3, generating the vector pHOG19-3. The PCR fragment of the V_L domain of anti-CD19 followed by a segment coding for a c-myc epitope and a hexahistidinyl tail was generated using the primers DP3, 5'-AGCACACACAAGCTTGGCGGTGATATCTTGCTCACCCAAA-CTCCA plus DP4, 5'-AGCACACTCTAGAGACACACAGATCTT TAGTGATGGTATGGTATGAGTTAGG. The PCR frag-

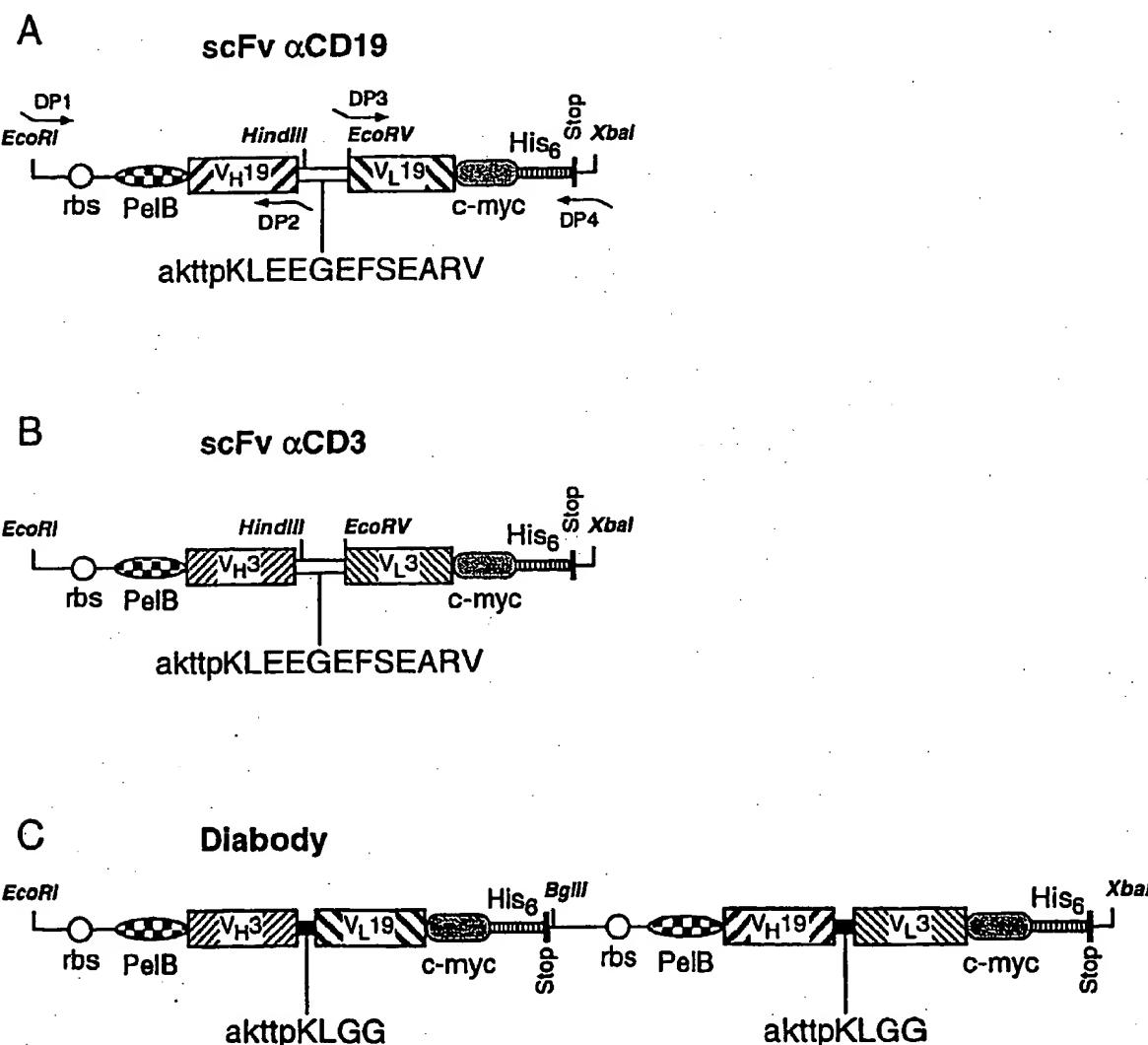


FIGURE 1—Schematic representation of operons encoding anti-CD19 and anti-CD3 scFvs in plasmid pHOG21 (Kipriyanov *et al.*, 1997a) (*a* and *b*, respectively) and dicistronic operon encoding the bispecific anti-human CD3 \times CD19 diabody in plasmid pKID3 \times 19 (*c*). The positions of primers and most important restriction sites used for constructing the plasmids are shown. The locations of ribosome binding sites (rbs), pelB leader sequences (pelB), c-myc epitopes (c-myc), hexahistidinyl tags (His₆) and stop codons (Stop) are indicated. Amino acid sequences of linkers connecting V-domains are shown below each drawing. In the linker, amino acids derived from the C_{H1} domain are indicated by small letters and residues introduced artificially are shown in block letters.

ment of the V_L domain of anti-CD19 contained a *Bgl*II site near the 3' end of the coding strand. It was digested with *Hind*III and *Xba*I and ligated with the *Hind*III/*Xba*I linearized plasmid pHOG-dmOKT3, generating the vectors pHOG3-19. The expression plasmid pKID3 X 19 for cosecretion of the two hybrid scFvs was constructed by ligation of the *Bgl*II/*Xba*I restriction fragment from pHOG3-19 comprising the vector backbone and the *Bgl*II/*Xba*I fragment from pHOG19-3. All sequences encoding hybrid scFv fragments were verified by the dideoxynucleotide method (Sanger *et al.*, 1977).

ScFv and diabody expression and purification

Bacterial growth, induction and isolation of periplasmic extracts was performed as previously described (Kipriyanov *et al.*, 1996, 1997a, b). For isolation of anti-CD3 and anti-CD19 scFv, the culture supernatant and the soluble periplasmic extract were combined and concentrated using Amicon YM10 membranes with a 10 kDa cut-off (Amicon, Witten, Germany) followed by thorough dialysis against 50 mM Tris-HCl, 1 M NaCl, pH 7.0. The diabody was concentrated by ammonium sulfate precipitation (final concentration 70% of saturation) as recommended (Atwell *et al.*, 1996). The protein precipitate was collected by centrifugation (30,000g, 4°C, 30 min) and dissolved in 1/10 of the initial volume of 50 mM Tris-HCl, 1 M NaCl, pH 7.0. Purification was achieved by IMAC as previously described (Kipriyanov *et al.*, 1997a). The final purification of scFv- α CD3 ($I_a = 7.52$) and scFv- α CD19 ($I_a = 6.19$) was achieved by ion-exchange chromatography on a MonoS HR5/5 column (Pharmacia) in 50 mM MES, pH 6.0, or on a MonoQ HR5/5 column (Pharmacia) in 20 mM Tris-HCl, pH 8.0, respectively, with a linear 0–1 M NaCl gradient. The purified antibody preparations were dialyzed against PBS (15 mM Na-phosphate, 0.15 M NaCl, pH 7.0). All purification procedures were performed at 4°C. For long-time storage, diabody and scFv were frozen in the presence of BSA (final concentration 10 mg/ml) or 10% FCS and stored at –80°C.

Measurement of protein concentration

Protein concentrations were determined by the Bradford (1976) dye-binding assay, using the Bio-Rad (Munich, Germany) protein assay kit. The concentrations of purified diabody, scFv- α CD3 and scFv- α CD19 were determined from the A_{280} values using the extinction coefficients $\epsilon^{1\text{mg/ml}} = 1.89, 1.84$ and 1.82 , respectively, calculated according to Gill and von Hippel (1989).

SDS-PAGE and size-exclusion chromatography

SDS-PAGE was performed according to Laemmli (1970) under reducing conditions. Western blot analyses using either rabbit serum A (Breitling *et al.*, 1991) recognizing the N-terminus of the processed antibody fragment (without a *peB* leader) or mouse MAb 9E10 (IC Chemikalien, Ismaning, Germany) specific for a peptide of the c-myc oncogene were performed as previously described (Kipriyanov *et al.*, 1994). Analytical gel filtration of the diabody and scFv preparations was performed in PBS using a Superdex 75 HR10/30 column (Pharmacia). Sample volume and flow rate were 200 μ l and 0.5 ml/min, respectively. The column was calibrated with a low molecular weight gel filtration calibration kit (Pharmacia).

Flow cytometry

The human CD3 $^+$ /CD19 $^-$ acute T cell leukemia line Jurkat and the CD19 $^+$ /CD3 $^-$ B cell line JOK-1 were used for flow cytometry, performed as previously described (Kipriyanov *et al.*, 1996). In brief, 5×10^5 cells in 50 μ l RPMI 1640 medium (GIBCO BRL, Eggenstein, Germany) supplemented with 10% FCS and 0.1% sodium azide (referred to as complete medium) were incubated with 100 μ l of a recombinant antibody preparation for 45 min on ice. After washing with complete medium, the cells were incubated with 100 μ l of 10 μ g/ml anti c-myc MAb 9E10 (IC Chemikalien) in the same buffer for 45 min on ice. After a second washing cycle, the cells were incubated with 100 μ l of FITC-labeled goat anti-mouse IgG (GIBCO BRL) under the same conditions as before. The cells

were then washed again and resuspended in 100 μ l of 1 μ g/ml solution of propidium iodide (Sigma, Deisenhofen, Germany) in complete medium to exclude dead cells. The relative fluorescence of stained cells was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Affinity determination

Affinities of MAb HD37 (Pezzutto *et al.*, 1987) and scFv- α CD19 (Kipriyanov *et al.*, 1996) were determined by cellular RIA. Antibody (100 μ g) was labeled with 1 mCi [125 I]-iodide by the chloramine T method (Greenwood *et al.*, 1963). RIA was performed in a flexible polyvinyl chloride microtiter plate blocked with PBS containing 0.2% (w/v) gelatin at 4°C overnight. After washing, 10 6 JOK-1 cells in 50 μ l PBS containing 0.2% gelatin and 5% (v/v) pooled human IgG (Venimun Behringwerke, Marburg, Germany) were incubated in triplicate with increasing amounts of the radiolabeled antibody preparation for 1 hr at room temperature. The plate was washed and aspirated 3 times using PBS/0.2% gelatin. The dried plate was sliced, and the radioactivity in individual wells was measured using a gamma-counter. Affinity constants were determined by a Scatchard (1949) plot analysis.

Apparent affinities of diabody and scFv were determined from competitive inhibition assays as previously described (Kipriyanov *et al.*, 1997b). In brief, increasing concentrations of purified antibody fragment were added to a subsaturating concentration of FITC-labeled MAb OKT3 or HD37 and incubated with Jurkat or JOK-1 cells, respectively, as described above for FACScan analysis. Fluorescence intensities of stained cells were measured as described above. Binding affinities were calculated according to the following equation derived from that of Schodin and Kranz (1991):

$$K_{d(I)} = (1 + [FITC-MAb] \times K_{d(1)}) / IC_{50}$$

where I is the unlabeled inhibitor (diabody or scFv), [FITC-MAb] is the concentration of FITC-labeled MAb, $K_{d(1)}$ is the binding affinity of MAb and IC_{50} is the concentration of inhibitor that yields 50% inhibition of binding. Affinity constant values of 1.2×10^9 M $^{-1}$ and 2.5×10^9 M $^{-1}$ were taken for MAb OKT3 (Adair *et al.*, 1994) and HD37 (determined by RIA), respectively.

Analyses of diabody and scFv stability

The antibody fragments were stored in freshly prepared human serum from a healthy donor at 37°C at a concentration 20 μ g/ml. At given time points, 250 μ l aliquots were taken under sterile conditions, frozen and kept at –80°C. Activities of samples after storage were determined by flow cytometry.

Preparation and stimulation of effector cells

Human PBMCs were isolated from the buffy coat of healthy donors by Ficoll/Hypaque (Pharmacia) density gradient centrifugation. The PBMC interphase was washed twice in PBS and used immediately as effector cells. Cultures of PBMC were grown using RPMI 1640 (GIBCO BRL) supplemented with 2% heat inactivated FCS (GIBCO BRL), 2 mM glutamine, 1 mM pyruvate and 0.05 mM 2-ME. To obtain CTLs, PBMCs were cultured at a concentration of 2×10^6 ml in medium containing anti-CD3 MAb OKT3 (5 μ g/ml) and recombinant human IL-2 (20 U/ml) (EuroCetus, Amsterdam, The Netherlands). After 4 days, the cells were washed twice to remove remaining antibody and cultured overnight in medium alone. The cytotoxicity assay was performed on day 5.

Cytotoxicity assay

The CD19-expressing Burkitt's lymphoma cell lines Raji and Namalwa were used as target cells. Cells were cultured in RPMI 1640 (GIBCO BRL) supplemented with 10% heat inactivated FCS (GIBCO BRL), 2 mM glutamine and 1 mM pyruvate at 37°C in a humidified atmosphere containing 7.5% CO₂. The cytotoxic T cell assays were carried out in RPMI 1640 medium supplemented with 10% FCS, 10 mM HEPES, 2 mM glutamine, 1 mM pyruvate and 0.05 mM 2-ME. Cytotoxic activity was assessed using a standard

[⁵¹Cr]-release assay: 2×10^6 target cells were labeled with 200 μ Ci Na[⁵¹Cr]O₄ (Amersham-Buchler, Braunschweig, Germany) followed by 4 washing cycles and resuspended in medium at a concentration 2×10^6 /ml. Effector cells were adjusted to a concentration of 5×10^6 ml. Increasing amounts of CTLs in 100 μ l were titrated to 10^4 target cells/well in 50 μ l. Antibodies (50 μ l) were added to each well. The whole assay was set up in triplicate and incubated for 4 hr at 37°C. Supernatant (100 μ l) was harvested and assayed for [⁵¹Cr] release in a gamma-counter (Cobra Auto Gamma; Canberra Packard, Dreieich, Germany). Maximum release was determined by incubating the target cells in 10% SDS, and spontaneous release was determined by incubating the cells in medium alone. Specific lysis (%) was calculated as: (experimental release - spontaneous release)/(maximal release - spontaneous release) $\times 100$.

RESULTS

Diabody design and construction

Single-chain Fv fragments derived from the hybridomas HD37 (Pezzutto *et al.*, 1987) and OKT3 (Kung *et al.*, 1979) were used for creating a bispecific CD3 \times CD19 diabody. A significant increase in the stability of the OKT3 anti-CD3 scFv was achieved by substituting a serine for cysteine in position 100A of the V_H domain (Kipriyanov *et al.*, 1997b). The C-terminus of a V_L domain was connected to the N-terminus of another specificity using a short rigid linker to restrict intra-chain pairing of V_H and V_L. The linker design was based on our strategy of cloning antibody V_H domains using an anti-sense primer complementary to the 5'-region of the γ chain C_{μ1} domain gene (Kipriyanov *et al.*, 1996, 1997b). Seven amino acids introduced by this primer formed a major part of the linker (Fig. 1). The plasmid pKD3 \times 19 was constructed to express the CD3 \times CD19 diabody by co-secretion of the 2 hybrid scFvs V_{H3}-V_{L19} and V_{H19}-V_{L3} from a dicistronic operon (Fig. 1). The operon is under the transcriptional control of the *wi lac* promoter/operator, which is inducible with IPTG. Each hybrid scFv is preceded by a *peB* leader sequence to direct secretion to the periplasmic space of *E. coli*. Both hybrid scFv genes are followed by nucleotide sequences coding for a c-myc tag for immunodetection and a hexahistidinyl tail for purification of recombinant product using IMAC (Fig. 1).

Diabody expression and purification

The CD3 \times CD19 diabody was secreted from *E. coli* cells transformed with the pKD3 \times 19 plasmid. Relatively equal amounts of soluble hybrid scFv V_{H3}-V_{L19} and V_{H19}-V_{L3} were detected in crude periplasmic extracts by Western blot analysis using either MAb 9E10 specific to the C-terminal c-myc epitope or by serum A specific for the N-terminus of processed (without *peB* leader) antibody fragment (data not shown). FACScan analyses of crude periplasmic extracts demonstrated that the diabody produced by bacteria specifically interacted with both CD3-positive Jurkat cells and CD19-positive JOK-1 cells. In contrast, neither hybrid scFv alone bound these cell lines (data not shown).

To obtain higher yields of soluble antibody fragments, we added 0.4 M sucrose to bacterial cells that had been induced with IPTG. We have shown that under these conditions the yield of soluble scFv can be increased up to 150-fold (Kipriyanov *et al.*, 1997a). The diabody was found to accumulate in the *E. coli* periplasm and was also released into the culture medium (data not shown). After concentrating by ammonium sulfate precipitation and purification by IMAC, yields of 2.0–2.5 mg/l with a purity greater than 95% were achieved (Fig. 2a). In contrast, ammonium sulfate was shown to be rather ineffective for precipitating monospecific anti-CD19 and anti-CD3 single chain Fv fragments that were isolated in parallel. Much higher yields of the purified scFv antibody fragments were obtained by concentrating by ultrafiltration prior to IMAC (data not shown).

Analyses of diabody and scFv by SDS-PAGE and size-exclusion FPLC

Purified antibody fragments were analyzed by electrophoresis on 12% SDS polyacrylamide gels. Under these conditions, the diabody was resolved into 2 protein bands corresponding to the calculated M_r of 28,900 for scFv V_{H19}-V_{L3} and 29,300 for scFvs V_{H3}-V_{L19} (Fig. 2a). Anti-CD3 and anti-CD19 scFvs appeared as single bands (calculated M_r are 29,800 and 30,400, respectively).

An analysis of antibody fragments by gel-filtration on a Superdex 75 column is shown in Figure 2b. The scFv-αCD3 consisted only of monomers, the scFv-αCD19 of monomers, dimers and a multimeric form and the bispecific diabody only of dimers. The apparent m.w. of the diabody deduced from its chromatographic mobility was lower than its actual m.w., reflecting its compact structure (Perisic *et al.*, 1994).

Antigen binding specificity and affinity of diabody and scFv

Flow cytometry experiments demonstrated a specific interaction of the diabody with both human CD19-positive JOK-1 and CD3-positive Jurkat cells. The fluorescence intensities were fairly comparable to those obtained using the parental monospecific scFvs at similar concentrations (Fig. 3).

The CD19 and CD3 binding affinities of the diabody were estimated by competitive binding to human JOK-1 and Jurkat cells in the presence of either FITC-labeled MAb HD37 (anti-CD19) or OKT3 (anti-CD3). The IC₅₀ value of diabody competing with FITC-OKT3 for binding T-cells was similar to that of scFv-αCD3 (Fig. 4b; Table I). In contrast, scFv anti-CD19 competed with FITC-HD37 more effectively than the bispecific diabody (Fig. 4a), probably because a significant amount of this scFv fragment was in a dimeric and multimeric form (Fig. 2b). Direct affinity measurements of radio-iodinated anti-CD19 MAb HD37 and scFv demonstrated that both have very similar affinity constants in the sub-nanomolar range (Table I), reflecting the bivalent nature of the scFv anti-CD19.

Table I summarizes the results of affinity measurements. The similarity in affinities of the bispecific diabody with the parental scFvs suggests that all the non-covalent heterodimers (diabody) are correctly formed by specific V_H-V_L interactions. The affinity of the diabody for CD19-positive target B cells was 10-fold higher than its affinity for CD3 positive effector T cells.

Stability of bispecific diabody in human serum

One might expect that the bispecific diabody would be rather labile, as it was formed by non-covalent interactions of 2 hybrid scFv molecules. We therefore investigated the stability of the diabody and both parental anti-CD3 and anti-CD19 scFvs when stored at a fairly low concentration in human serum at 37°C for prolonged periods of time. A concentration of 20 μ g/ml was chosen to avoid the fluorescence plateau in FACScan analysis (Kipriyanov *et al.*, 1997b). The residual antigen binding activity was estimated by flow cytometry. We found that the anti-CD3 scFv, which retained 70% of activity after 5 days of incubation, appeared to be significantly more stable than anti-CD19 scFv, which lost 50% of its activity after 36 hr of incubation and 95% after 4 days (Fig. 5). The diabody had an intermediate stability. It was less stable than the anti-CD3 scFv but slightly more stable than the anti-CD19 scFv (Fig. 5). The loss of both CD3 and CD19 binding had similar time kinetics, indicating an interdependent denaturation of the 2 binding domains.

Induction of specific cytotoxicity by diabody

The ability of the bispecific diabody to induce tumor cell lysis by redirecting T cell-mediated cytotoxicity was investigated using PBMC from healthy donors as effector cells. After stimulating with soluble MAb OKT3 and IL-2 for 4 days, the cells were washed to remove remaining antibody and cultured in medium alone overnight. Human B cell lines Raji and Namalwa expressing CD19 were used as target cells. The effect of the bispecific diabody was measured using a standard [⁵¹Cr]-release assay with increasing

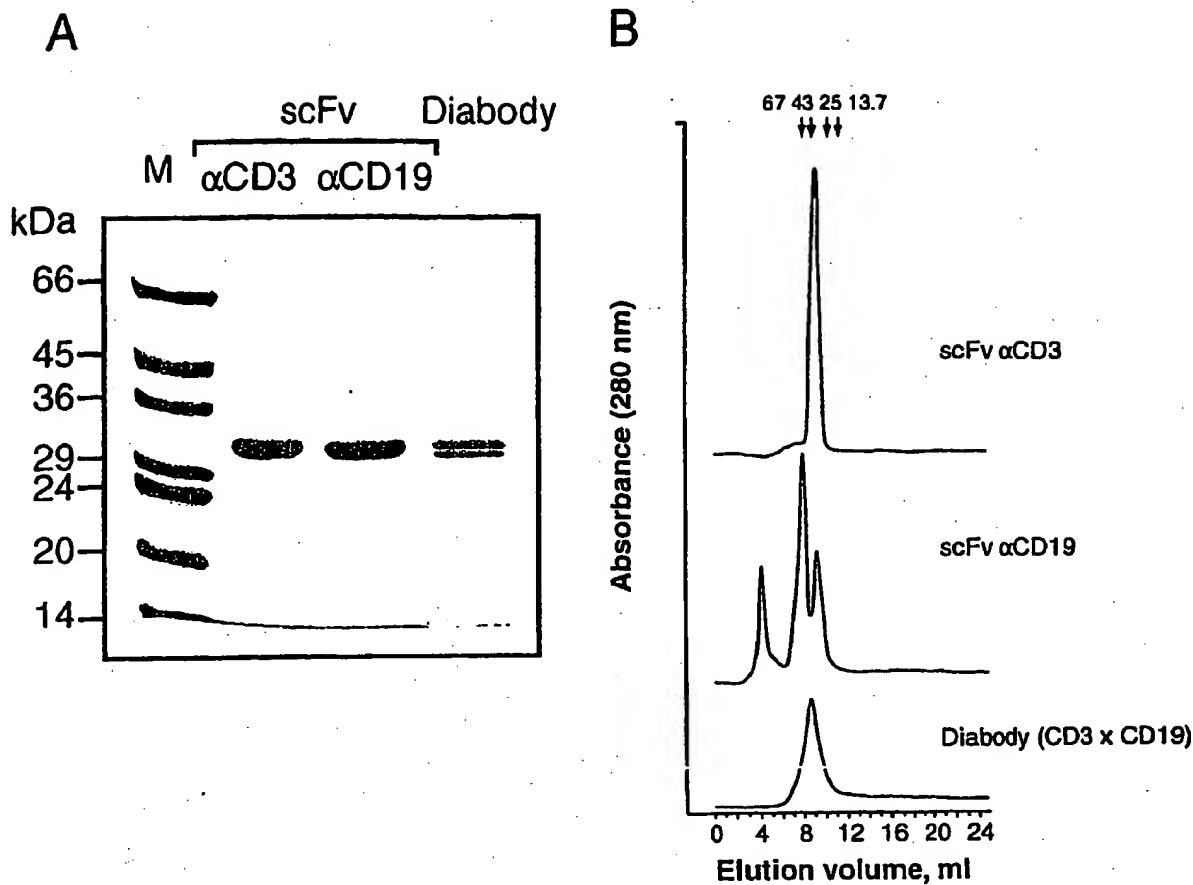


FIGURE 2 – Analyses of purified scFv anti-CD3, scFv anti-CD19 and bispecific diabody. (a) 12% SDS-PAGE under reducing conditions. The gel was stained with Coomassie brilliant blue. (b) Analytical gel filtration on a Superdex 75 column. The elution buffer was PBS, pH 7.0. Sample volume and flow rate were 200 μ l and 0.5 ml/min, respectively. Molecular masses were calibrated with BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

effector to target cell ratios. A mixture of both parental scFv fragments (anti-CD3 and anti-CD19) served as a negative control for determining the background lysis. Although the observed background lysis was different in different experiments, the diabody appeared to be quite potent in retargeting activated PBMC to lyse both target cell lines in a concentration-dependent manner (Fig. 6).

The CD3 X CD19 diabody was compared with a BsAb of the same specificity isolated from a hybrid-hybridoma OKT3 X HD37 (Csóka *et al.*, 1996). Bispecific diabody proved to be 10-fold more potent over the BsAb on a weight basis (3-fold on a molar basis) (Fig. 6a). Moreover, the increased cytotoxicity of the diabody was independent of the effector:target ratio (Fig. 6).

DISCUSSION

The 95 kDa CD19 antigen represents the broadest lineage-specific marker expressed on human B cells: it is present on the surface of virtually all B lymphocytes, including early B progenitor cells. CD19 is lost during the terminal stages of B cell differentiation but is exposed on the vast majority of B cell tumors (Uckun and Ledbetter, 1988). We therefore chose this antigen as the target molecule for BsAb-mediated cytotoxicity and constructed an anti-human CD19 scFv (Kipriyanov *et al.*, 1996).

Although different BsAb cross-linking NK cells (De Palazzo *et al.*, 1992) or activated neutrophils (Michon *et al.*, 1995) to tumor cells have been described, the most effective approach for tumor

rejection appears to be targeting via the CD3 molecule on cytotoxic T cells. The feasibility and effectiveness of this immunotherapeutic concept has been studied extensively in pre-clinical models, as well as in phase I clinical trials (Bolhuis *et al.*, 1996).

For the generation of a bispecific antibody suitable for therapy of human B cell malignancies, we aimed to construct a small recombinant molecule with dual specificity for both the human B cell surface antigen CD19 and the signal-transducing CD3 ϵ chain of the human TCR/CD3 complex. Although numerous anti-human CD3 MAbs have been used to study the T cell activation (Schwinzer *et al.*, 1992) and to create BsAb either by chemical conjugation (Anderson *et al.*, 1992; Nitta *et al.*, 1990) or by the quadroma technique (Bohlen *et al.*, 1993; Jacobs *et al.*, 1997), only 2 different anti-human CD3 antibody fragments have been used to date for the creation of recombinant BsAb. Variable domains derived from the hybridoma TR66 were used for constructing CD4-FvCD3 bispecific single chain molecules Janusins (Trauecker *et al.*, 1991) and later for the creation of (scFv)₂ specific for both the epithelial 17-1A antigen and human CD3 (Mack *et al.*, 1995). In the second case, humanized V domains originally derived from the murine hybridoma UCHT1 were used for constructing anti-CD3/anti-p185^{HER2} F(ab')₂ (Shalaby *et al.*, 1992) and a diabody (Zhu *et al.*, 1996). For constructing the BsAb described here, we used an scFv gene derived from the well-known hybridoma OKT3, which had been modified to improve its stability and folding in bacteria (Kipriyanov *et al.*, 1997b).

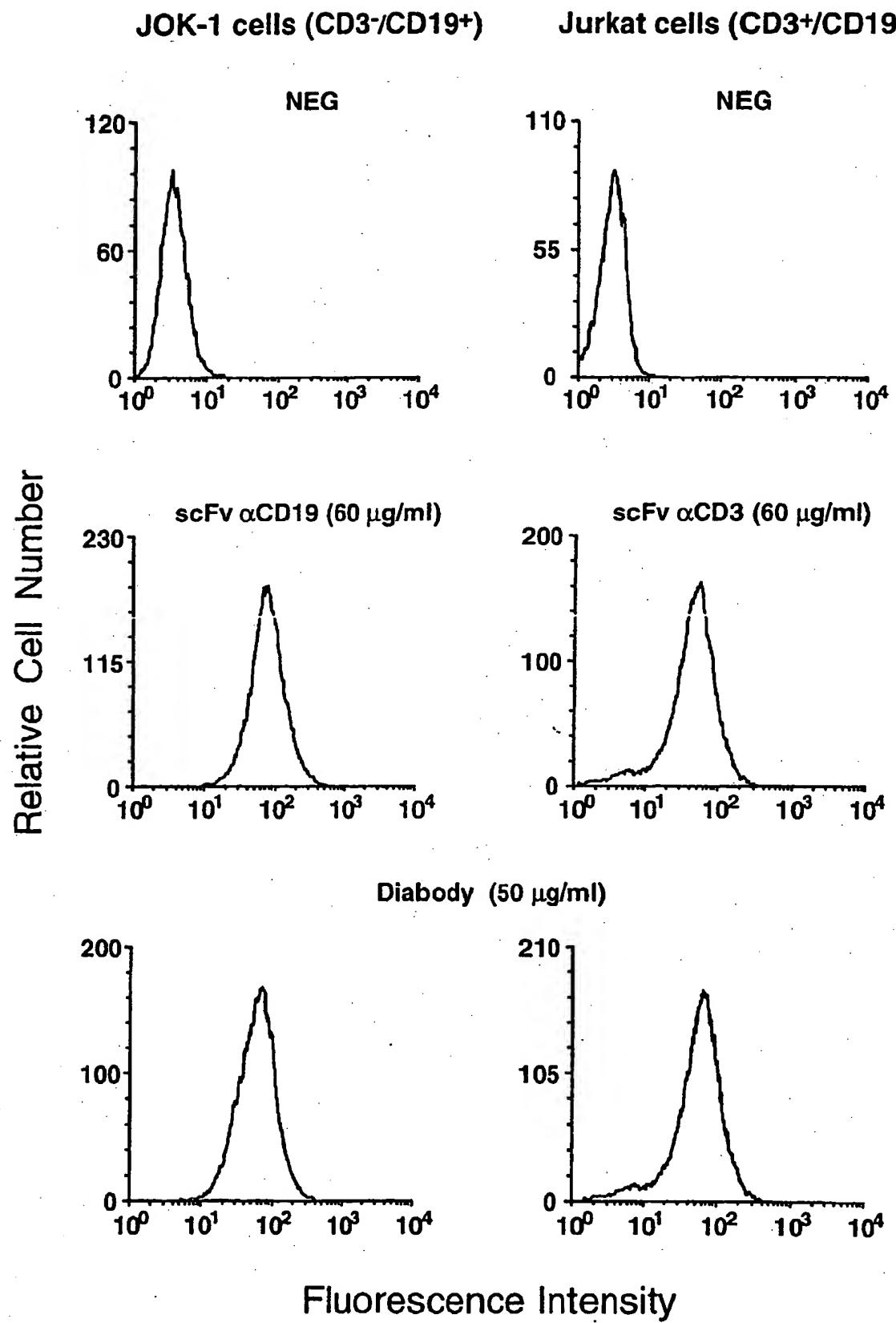


FIGURE 3 – Flow cytometric analysis of scFv and diabody binding to CD19⁺/CD3⁻ JOK-1 cells and CD3⁺/CD19⁻ Jurkat cells. As a negative control, the binding of an irrelevant scFv was used.

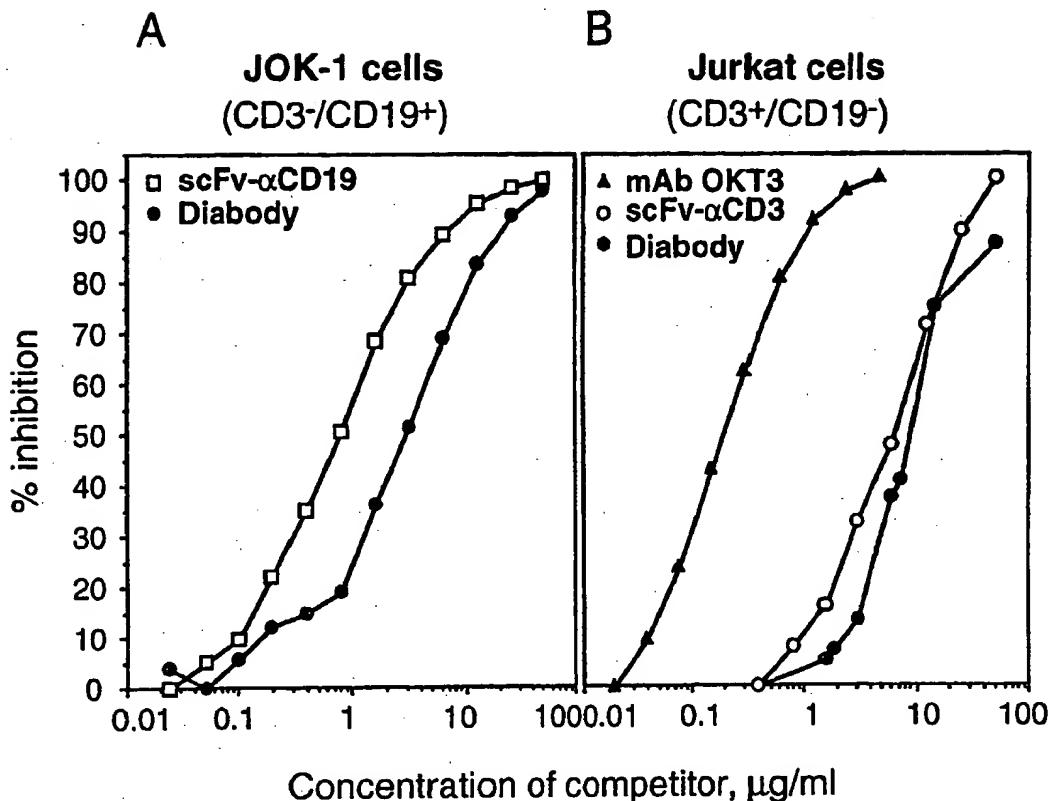


FIGURE 4 – Analyses of apparent affinities by flow cytometry. Inhibition of binding of FITC-labeled MAbs HD37 to JOK-1 cells (*a*) and inhibition of binding of FITC-labeled MAbs OKT3 to Jurkat cells (*b*) in the presence of MAb OKT3, scFv-αCD19, scFv-αCD3 and bispecific diabody are shown.

TABLE I – AFFINITIES OF MAb AND ANTIBODY FRAGMENTS

Antibody	IC ₅₀ (nM)	K _a (M ⁻¹) ¹	K _s (M ⁻¹) ²
<i>JOK-1 cells</i>			
MAb HD37	n.d. ³	n.d.	2.5 × 10 ⁹
scFv-αCD19	24.0	9.7 × 10 ⁸	2.9 × 10 ⁹
Diabody	48.8	4.8 × 10 ⁸	n.d.
<i>Jurkat cells</i>			
MAb OKT3	1.3	7.7 × 10 ⁸	n.d.
scFv-αCD3	222.2	4.5 × 10 ⁷	n.d.
Diabody	156.0	6.4 × 10 ⁷	n.d.

¹Affinities determined from inhibition experiments. ²Affinities determined from Scatchard plots. ³Not determined.

Initial attempts to produce bispecific CD3 × CD19 (scFv)₂ molecules, as recommended by Gruber *et al.* (1994), were unsatisfactory because of the small yields and low anti-CD3 activity (data not shown). We therefore constructed a more rigid cross-over scFv dimer (diabody). This construct has been shown to have 2 antigen-binding sites at opposite ends of the molecule, separated by 65 Å, which is sufficient to span the distance between 2 cells (Holliger *et al.*, 1996; Perisic *et al.*, 1994). The CD3 × CD19 diabody was produced by bacteria in a soluble functional form and could be retrieved from periplasmic extracts and culture medium in one step with a yield comparable to those obtained for parental scFv fragments.

Structurally, the isolated diabody was a stable compact dimer with an apparent m.w. around 50 kDa. Theoretically, the co-secretion of two hybrid scFv fragments may give rise to 2 types of dimer: active heterodimers and likely inactive homodimers. Affinity measurements indicated that the diabody was mostly, if not

completely, in the active heterodimeric form. It bound to human CD3 with an affinity indistinguishable from that of the parental scFv-αCD3 and to human CD19 with an affinity about one half that of scFv-αCD19. This latter scFv has been shown to have a very high affinity in the subnanomolar range, similar to that of the parental MAb. Size-exclusion chromatography demonstrated that a significant part of this scFv was present in the form of non-covalent dimers and even higher oligomers, probably resulting in an enhanced affinity due to the increased avidity. In contrast, the CD19 binding domain of the diabody is only monomeric.

The higher affinity of the anti-CD19 moiety resulted in an almost 10-fold stronger binding of the diabody to the surface of target B cells than to the surface of T cells. Strong binding to a target tumor cell and weaker binding to an effector cell may have certain advantages for cancer therapy. For example, experiments *in vitro* demonstrated that the cytotoxic potential of recombinant BsAb does not depend on the affinity of its CD3-binding domain (Zhu and Carter, 1995). Furthermore, in a model of TCR serial triggering (Vilimutti *et al.*, 1995), a high off-rate of the TCR is essential since it allows a single peptide-MHC complex to engage many TCRs in successive rounds of ligation, triggering and dissociation. Therefore, a higher affinity could result in less stimulation because the lower off-rate may prevent TCR reuse. A similar situation may also be present in the case of the surrogate antigenic stimulation of T cells through the anti-CD3 part of BsAb. For example, i.v. administration of a F(ab')₂ of OC/TR BsAb, which has a relatively high affinity for human CD3e (10⁸ M⁻¹) (Jacobs *et al.*, 1997), induced a generalized *in vivo* activation with severe side effects (Tibben *et al.*, 1993), which most probably originated from target cell-independent direct activation of T cells and subsequent release of cytokines. Therefore, a recombinant BsAb that binds strongly to

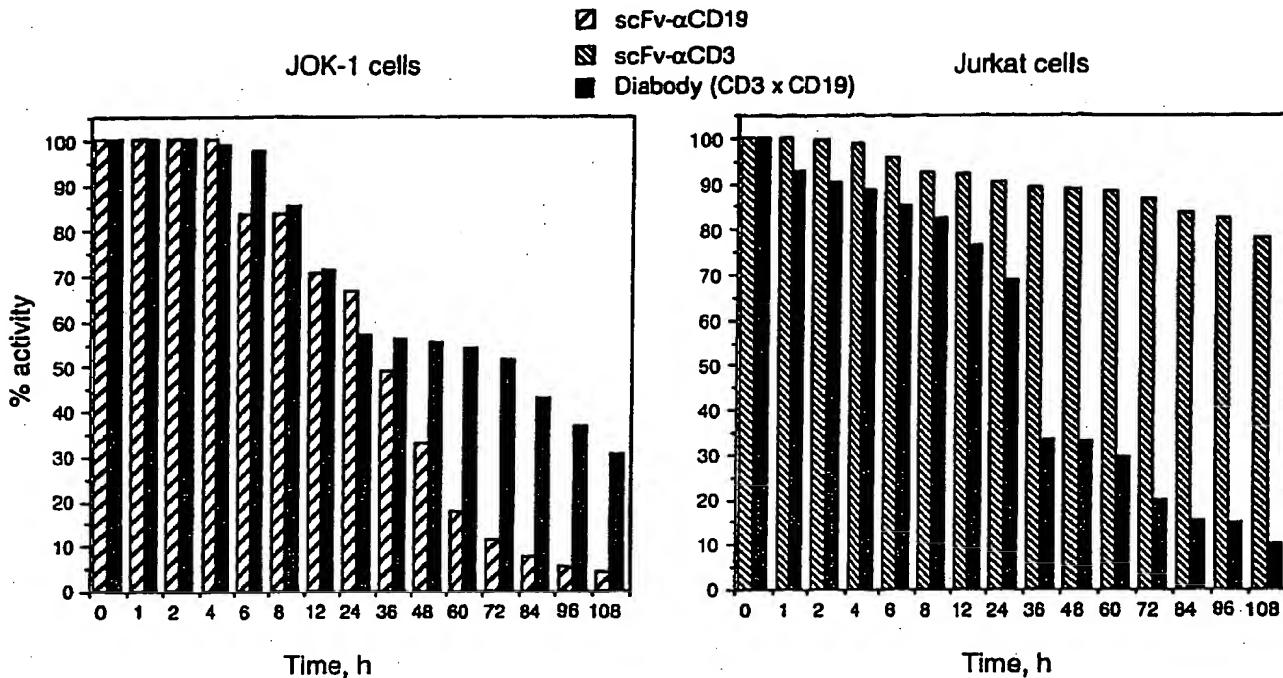


FIGURE 5 – Stability of scFv fragments and diabody in human serum at 37°C. The antibody fragments were incubated at 20 μ g/ml in human serum at 37°C for the times shown. The CD19 and CD3 binding activity was assessed by flow cytometry using CD19 $^{+}$ /CD3 $^{-}$ JOK-1 and CD3 $^{+}$ /CD19 $^{-}$ Jurkat cells. Activity of the samples at point zero was taken as 100%.

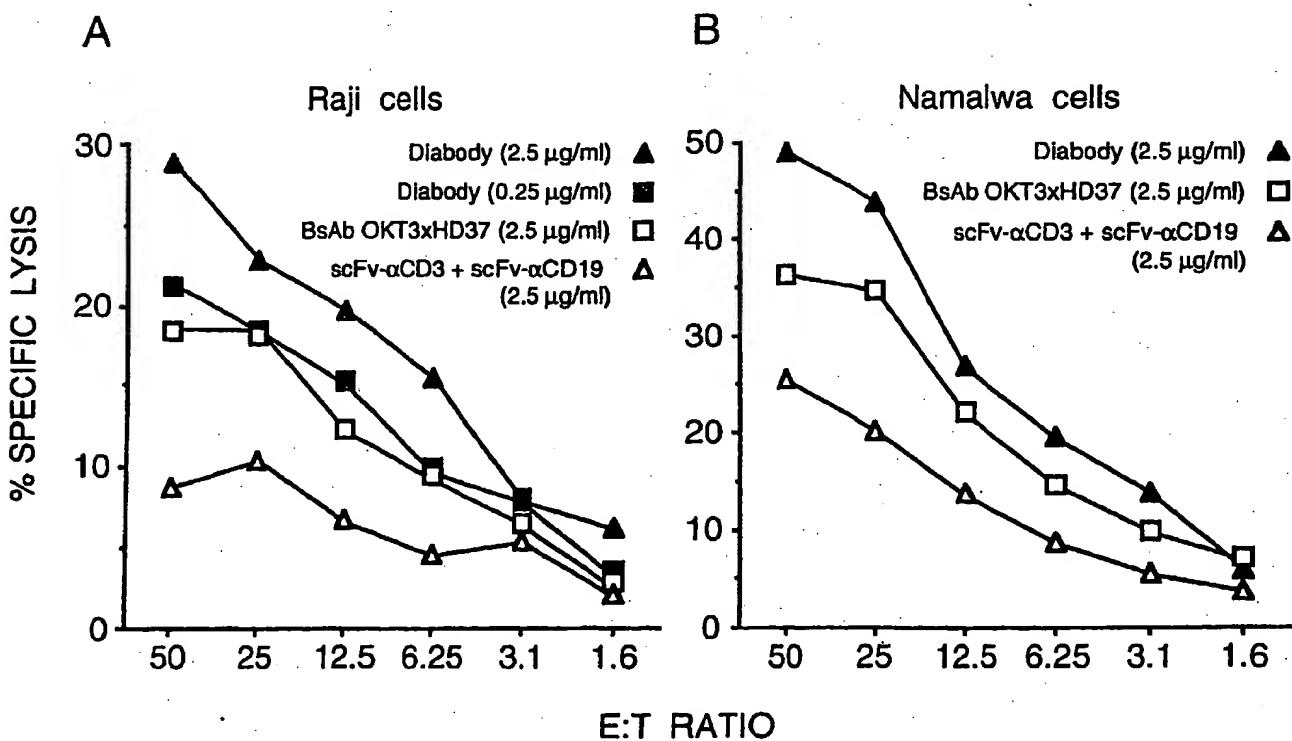


FIGURE 6 – Diabody-mediated lysis of CD19-positive B cells by activated human PBMC. The human CD19 expressing [31 Cr]-labeled target cell lines Raji (a) and Namalwa (b) were co-incubated with effector CTLs at different effector/target ratios for 4 hr. Percent cytotoxicity was calculated based on [31 Cr]-release in the presence of 2.5 μ g/ml of parental scFvs, 0.25 μ g/ml of diabody, 2.5 μ g/ml of diabody or 2.5 μ g/ml of BsAb OKT3 \times HD37.

target tumor cells and significantly more weakly to the signal-transducing CD3 ϵ chain of the TCR/CD3 complex on effector cells may prove to have the most desirable properties.

An analysis of the stability of antibody fragments in human serum demonstrated a significant difference between scFv- α CD3 and scFv- α CD3. The present data confirm our previous observation that scFv derived from the hybridoma OKT3 and containing a Cys/Ser substitution in CDR-H3 has an enhanced stability (Kipriyanov *et al.*, 1997b). A comparison of the stabilities of the diabody and the parental scFvs, performed here suggests a stabilizing effect of the anti-CD3 moiety of the diabody on the CD19-specific moiety and also an interdependent denaturation of the 2 binding domains.

The bispecific diabody was able to induce an efficient lysis of the target cells by human CTLs. A comparison with a hybrid-hybridoma BsAb of the same dual specificity (Csóka *et al.*, 1996) indicated that the diabody was even more potent in recruiting activated T cells for cytolysis of the target cells. The increased

efficiency of the smaller diabody may be due to the closer proximity of the target cell and T cells connected by the diabody (Holliger *et al.*, 1996; Perisic *et al.*, 1994).

The prohibitive cost of preparing bispecific antibodies with the quadroma technology has made it difficult to start clinical trials. Recombinant antibodies, on the other hand, can be produced cheaply in large quantities using high cell density fermentation of bacterial cultures (Horn *et al.*, 1996; Zhu *et al.*, 1996). Furthermore, these smaller antibodies are less immunogenic due to the absence of constant domains. Consequently, we are now concentrating on the production of larger quantities of the diabody for testing in phase I clinical trials.

ACKNOWLEDGEMENTS

We thank Mr. A. Keller for excellent technical assistance.

REFERENCES

ADAIR, J.R., ATHWAL, D.S., BODMER, M., BRIGHT, S.M., COLLINS, A., PULITO, V.L., RAO, P.E., REEDMAN, R., ROTHERMEL, A.L., XU, D., ZIVIN, R.A. and JOLLIFFE, L.K., Humanization of the murine anti-human CD3 monoclonal antibody OKT3. *Hum. Antibodies Hybridomas*, 5, 41-47 (1994).

ANDERSON, P.M., CRIST, W., HASZ, D., CARROLL, A.J., MYERS, D.E. and UCKUN, F.M., G19.4(α CD3) X B43(α CD19) monoclonal antibody heteroconjugate triggers CD19 antigen-specific lysis of t(4;11) acute lymphoblastic leukemia cells by activated CD3 antigen-positive cytotoxic T cells. *Blood*, 80, 2826-2834 (1992).

ATWELL, J.L., PEARCE, L.A., LAH, M., GRUEN, L.C., KORTT, A. and HUDSON, P., Design and expression of a stable bispecific scFv dimer with affinity for both glycoprotein and N9 neurominidase. *Mol. Immunol.*, 33, 1301-1312 (1996).

BIRD, R.E., HARDMAN, K.D., JACOBSON, J.W., JOHNSON, S., KAUFMAN, B.M., LEE, S.-M., LEE, T., POPE, S.H., RIORDAN, G.S. and WHITLOW, M., Single-chain antigen-binding proteins. *Science*, 242, 423-426 (1988).

BOHLEN, H., HOPPE, T., MANZKE, O., ENGERST, A., KUBE, D., WICKRAMANYAKE, P.D., DIEHL, V. and TESCH, H., Lysis of malignant B cells from patients with B-chronic lymphocytic leukemia by autologous T cells activated with CD3 X CD19 bispecific antibodies in combination with bivalent CD28 antibodies. *Blood*, 82, 1803-1812 (1993).

BOLHUIS, R.L.H., HOOGENDOORN, H.R. and GRATAMA, J.W., Targeting of peripheral blood T lymphocytes. *Springer Semin. Immunopathol.*, 18, 211-226 (1996).

BRADFORD, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72, 248-254 (1976).

BREITLING, F., DÜBEL, S., SEEHAUS, T., KLEWININGHAUS, I. and LITTLE, M., A surface expression vector for antibody screening. *Gene*, 104, 147-153 (1991).

BRENNAN, M., DAVIDSON, P.F. and PAULUS, H., Preparation of bispecific antibodies by chemical recombination of monoclonal immunoglobulin G1 fragments. *Science*, 229, 81-83 (1985).

CANEVARI, S. and 17 others, Regression of advanced ovarian carcinoma by intraperitoneal treatment with autologous T lymphocytes retargeted by a bispecific monoclonal antibody. *J. nat. Cancer Inst.*, 87, 1463-1469 (1995).

CARTER, P., RIDGWAY, J. and ZHU, Z., Toward the production of bispecific antibody fragments for clinical applications. *J. Hematother.*, 4, 463-470 (1995).

CsóKA, M., STRAUSS, G., DEHATIN, K.-M. and MOLDENHAUER, G., Activation of T cell cytotoxicity against autologous common acute lymphoblastic leukemia (cALL) blasts by CD3 X CD19 bispecific antibody. *Leukemia*, 10, 1765-1772 (1996).

DE PALAZZO, I.G., HOLMES, M., GARCIA-TAYLOR, C. and WEINER, L.M., Antitumor effect of a bispecific antibody targeting CA19-9 antigen and CD16. *Cancer Res.*, 52, 5713-5719 (1992).

FANGER, M.W., MORGANELLI, P.M. and GUYKE, P.M., Bispecific antibodies. *Crit. Rev. Immunol.*, 12, 101-124 (1992).

GILL, S.C. and VON HIPPEL, P.H., Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.*, 182, 319-326 (1989).

GREENWOOD, F.C., HUNTER, W.M. and GLOVER, J.C., The preparation of 131 I-labeled human growth hormone of high specific radioactivity. *J. Biochem.*, 89, 114-120 (1963).

GROSSBARD, M.L., PRESS, O.W., APPELBAUM, F.R., BERNSTEIN, I.D. and NAIDLER, L.M., Monoclonal antibody-based therapies of leukemia and lymphoma. *Blood*, 80, 863-878 (1992).

GRUBER, M., SCHODIN, B.A., WILSON, E.R. and KRAZ, D.M., Efficient tumor cell lysis mediated by a bispecific single chain antibody expressed in *Escherichia coli*. *J. Immunol.*, 152, 5368-5374 (1994).

HOLLIGER, P., BRISBIN, J., WILLIAMS, R.L., THIELEMANS, K. and WINTER, G., Specific killing of lymphoma cells by cytotoxic T-cells mediated by a bispecific diabody. *Protein Eng.*, 9, 299-305 (1996).

HOLLIGER, P., PROSPERO, T. and WINTER, G., "Diabodies": small bivalent and bispecific antibody fragments. *Proc. nat. Acad. Sci. (Wash.)*, 90, 6444-6448 (1993).

HORN, U., STRITTMATTER, W., KREBBER, A., KNUPFER, U., KUIAU, M., WENDEROTH, R., MÜLLER, K., MATZKU, S., PLÜCKTHUN, A. and RIESENBERG, D., High volumetric yields of functional dimeric miniantibodies in *Escherichia coli*, using an optimized expression vector and high-cell-density fermentation under non-limited growth conditions. *Appl. Microbiol. Biotechnol.*, 46, 524-532 (1996).

HUSTON, J.S., MUGGETT HUNTER, M., TAI, M.S., McCARTNEY, J., WARREN, F., HABER, E. and OPPERMANN, H., Protein engineering of single-chain Fv analogs and fusion proteins. *Methods Enzymol.*, 203, 46-88 (1991).

JACOBS, N., MAZZONI, A., MEZZANZANICA, D., NEGRI, D.R.M., VALOTA, O., COLNAGHI, M.I., MOUTSCHE, M.P., BONIVER, J. and CANEVARI, S., Efficiency of T cell triggering by anti-CD3 monoclonal antibodies (mAb) with potential usefulness in bispecific mAb generation. *Cancer Immunol. Immunother.*, 44, 257-264 (1997).

KIPRIYANOV, S.M., DÜBEL, S., BREITLING, F., KONTERMANN, R.E. and LITTLE, M., Recombinant single-chain Fv fragments carrying C-terminal cysteine residues: production of bivalent and biotinylated miniantibodies. *Mol. Immunol.*, 31, 1047-1158 (1994).

KIPRIYANOV, S.M., KUPRIYANOVA, O.A., LITTLE, M. and MOLDENHAUER, G., Rapid detection of recombinant antibody fragments directed against cell-surface antigens by flow cytometry. *J. Immunol. Methods*, 196, 51-62 (1996).

KIPRIYANOV, S.M., MOLDENHAUER, G. and LITTLE, M., High level production of soluble single chain antibodies in small-scale *Escherichia coli* cultures. *J. Immunol. Methods*, 200, 69-77 (1997a).

KIPRIYANOV, S.M., MOLDENHAUER, G., MARTIN, A.C., KUPRIYANOVA, O.A. und LITTLE, M., Two amino acid mutations in an anti-human CD3 single chain Fv antibody fragment that affect the yield on bacterial secretion but not the affinity. *Protein Eng.*, 10, 445-453 (1997b).

KOSTELNY, S.A., COLE, M.S. and TSO, J.Y., Formation of a bispecific antibody by the use of leucine zippers. *J. Immunol.*, 148, 1547-1553 (1992).

KUNG, P.C., GOLSTEIN, G., REINHERZ, E.L. and SCHLOSSMAN, S.F., Monoclonal antibodies defining distinctive human T cell surface antigens. *Science*, 206, 347-349 (1979).

LAEMMLI, U.K., Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (Lond.)*, 227, 680-685 (1970).

MACK, M., RIETHMÜLLER, G. and KUFER, P. A small bispecific antibody construct expressed as a functional single-chain molecule with high tumor cell cytotoxicity. *Proc. Natl. Acad. Sci. (Wash.)*, **92**, 7021-7025 (1995).

MICHON, J., MOUTEL, S., BARBET, J., ROSET-LEMINONNE, J.-L., DEO, Y.D., FRIDMAN, W.H. and TEILLAUD, J.-L. *In vitro* killing of neuroblastoma cells by neutrophils derived from granulocyte colony stimulating factor treated cancer patients using an anti-disialoganglioside/anti-Fc γ RI bispecific antibody. *Blood*, **86**, 1124-1130 (1995).

MILSTEIN, C. and CUENLLA, A.C. Hybrid hybridomas and their use in immunohistochemistry. *Nature (Lond.)*, **305**, 537-540 (1983).

NITTA, T., SATO, K., YAGITA, H., OKUMURA, K. and ISHI, S. Preliminary trial of specific targeting therapy against malignant glioma. *Lancet*, **335**, 368-376 (1990).

PERUSIC, O., WEBB, P.A., HOLLIGER, P., WINTER, G. and WILLIAMS, R.L. Crystal structure of a diabody, a bivalent antibody fragment. *Structure*, **2**, 1217-1226 (1994).

PEZZUTTO, A., DÖRKEN, B., RABINOVITCH, P.S., LEDBETTER, J.A., MOLDENHAUER, G. and CLARK, E.A. CD19 monoclonal antibody HD37 inhibits anti-immunoglobulin-induced B cell activation and proliferation. *J. Immunol.*, **138**, 2793-2799 (1987).

SANGER, F., NICKLEN, S. and COULSON, R. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. (Wash.)*, **74**, 5463-5467 (1977).

SCATCHARD, G. The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.*, **51**, 660-672 (1949).

SCHODIN, B.A. and KRAZD, D.M. Binding affinity and inhibitory properties of a single-chain anti-T cell receptor antibody. *J. Biol. Chem.*, **268**, 25722-25727 (1993).

SCHWINZER, R., FRANKLIN, R.A., DOMENICO, J., RENZ, H. and GEIFAND, E.W. Monoclonal antibodies directed to different epitopes in the CD3-TCR complex induce different states of competence in resting human T cells. *J. Immunol.*, **148**, 1322-1328 (1992).

SHALABY, M.R., SHEPARD, H.M., PRESTA, L., RODRIGUES, M.L., BEVERLEY, P.C., FELDMANN, M. and CARTER, P. Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protooncogene. *J. exp. Med.*, **175**, 217-225 (1992).

TIBBEN, J.G., BOERMAN, O.C., CLAESSENS, R.A.M.J., CORSTENS, F.H.M., VAN DEUREN, M., DE MULDER, P.H.M., VAN DER MEER, J.W.M., KEUSER, K.G.G. and MASSUGER, L.F.A.G. Cytokine release in an ovarian carcinoma patient following intravenous administration of bispecific antibody OC/TCR F(ab')₂. *J. nat. Cancer Inst.*, **85**, 1003-1004 (1993).

TRAUNECKER, A., LANZAVECCHIA, A. and KARJALAINEN, K. Bispecific single chain molecules (Janusins) target cytotoxic lymphocytes on HIV infected cells. *EMBO J.*, **10**, 3655-3659 (1991).

UCKUN, F.M. and LEDBETTER, J.A. Immunobiologic differences between normal and leukemic human B-cell precursors. *Proc. Natl. Acad. Sci. (Wash.)*, **85**, 8603-8607 (1988).

VALUTTI, S., MÜLLER, S., CELLA, M., PADOVAN, E. and LANZAVECCHIA, A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature (Lond.)*, **375**, 148-151 (1995).

WINTER, G. and MILSTEIN, C. Man-made antibodies. *Nature (Lond.)*, **349**, 293-299 (1991).

ZHU, Z. and CARTER, P. Identification of heavy chain residues in a humanized anti-CD3 antibody important for efficient antigen binding and T cell activation. *J. Immunol.*, **155**, 1903-1910 (1995).

ZHU, Z., ZAPATA, G., SHALABY, R., SNEDECOR, B., CHEN, H. and CARTER, P. High level secretion of a humanized bispecific diabody from *Escherichia coli*. *Bio/Technology*, **14**, 192-196 (1996).

Efficient Tumor Cell Lysis Mediated by a Bispecific Single Chain Antibody Expressed in *Escherichia coli*¹

Meegan Gruber, Beth A. Schodin, Erik R. Wilson, and David M. Kranz²

Department of Biochemistry, University of Illinois, Urbana, IL 61801

Recent advances in the expression of Abs in *Escherichia coli* have raised the possibility that virtually any specificity can be obtained by either cloning Ab genes from characterized hybridomas or by de novo selection using Ab gene libraries. Bispecific Abs have been more difficult to engineer because of problems inherent in the proper folding and association of V_H and V_L domains. In this report, a model system for expressing and testing the activity of a single chain bispecific Ab was used. The Ab contained the V_H and V_L genes from the anti-TCR Ab 1B2 joined by a 25 amino acid residue linker to the V_H and V_L genes from the anti-fluorescein Ab 4420. The 57-kDa single chain bispecific Ab (scFv₂) was purified in a single step by affinity chromatography through a fluorescein column at a yield of 1 mg/L of bacterial culture. Despite the presence of 1B2 V regions at the NH₂-terminus and a 10-residue c-myc peptide at the COOH-terminus, the refolded protein had an affinity for fluorescein that was nearly identical with the monospecific single chain Ab. The scFv₂ also bound the TCR of the mouse CTL clone 2C and redirected the lysis of human tumor cells that had fluorescein covalently linked to their surface. Lysis was mediated at scFv₂ concentrations that were 100-fold lower than the concentrations of Ab that inhibited normal recognition by CTL 2C. These results show that single chain bispecific Abs can mediate CTL lysis of target cells without the immunosuppressive side effects associated with the use of anti-TCR Abs. *Journal of Immunology*, 1994, 152: 5368.

As first shown almost 10 years ago (1–3), bispecific Abs that react with the TCR/CD3 complex of a CTL and with an epitope of a target cell can mediate specific lysis of the target cell by the CTL. This approach to redirecting T cell-mediated lysis has now been tested with a variety of different tumor-specific Abs and effector/target cell combinations (4). In addition, the possible effectiveness of these agents in targeting tumor cells *in vivo* has been demonstrated with xenogenic tumor transplants in athymic mice (5). Encouraging results in a preliminary clinical trial of patients with malignant glioma have been reported using CD3/glioma bispecific Abs together with lymphokine-activated killer cells (6).

Despite the promise of bispecific Abs in tumor therapy, the optimum method of preparing clinically useful Abs has not been resolved. There are two general approaches that have proven successful in targeting cells *in vitro* (4). One

method is the production of quadromas that secrete disulfide-linked mixtures of heavy and light chains from two parental hybridomas of the desired specificity. The other method is the chemical linkage of two different Abs using intact Abs or Fab fragments prepared either by protease digestion or expression of the Ig genes in *Escherichia coli*. Covalent linkage of Abs has been accomplished through the use of bifunctional agents that react with free amines or sulfhydryls.

Each of these methods yields a heterogeneous mixture of Abs. For example, quadromas secrete nonactive mixtures of heavy and light chain pairs that must be separated from the active bispecific Ab. Chemically coupled Abs also have different forms of the bispecific Ab, depending on the site of covalent linkage. Because of these properties, it is difficult to obtain a homogenous preparation and the Abs are subject to low yields and to variations among different preparations.

Advances in the expression of single chain Ab genes in *E. coli* (7–10) and the recent success in isolating a non-covalently-linked bispecific Ab from *E. coli* (11) have provided a possible solution to the problems of chemical heterogeneity and yield. We report here the first example of a functional single chain bispecific Ab (scFv₂) expressed in *E. coli* that mediates lysis of target cells. The construction contained the V_H and V_L genes from an anti-TCR Ab

Received for publication December 21, 1993. Accepted for publication March 17, 1994.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by National Institutes of Health Grant AI24635 (D.M.K.). M.G. was generously supported by a Carle Cancer Center Research Scholarship.

² Address correspondence and reprint requests to Dr. David M. Kranz, Department of Biochemistry, University of Illinois, Urbana, IL 61801.

(12, 13) linked to the V_H and V_L genes from an anti-fluorescein Ab (7). The bispecific Ab could be purified in a single step using a fluorescein-Sepharose column, and the yield of active Ab (~1 mg/L of bacterial culture) suggested that this method should provide an economical source of bispecific Abs. The single chain bispecific Ab had an affinity for fluorescein that was similar to the monospecific single chain protein expressed in *E. coli*.

CTL-mediated lysis of fluorescein-coupled tumor cells was observed at concentrations (200 ng/ml to 20 μ g/ml) of bispecific Ab that did not inhibit recognition of a normal ligand by the effector T cells. Thus, bispecific Abs could specifically target cells for lysis without suppressing a normal cytolytic T cell response.

Materials and Methods

Cell lines

The BALB.B-derived CTL clone 2C, which recognizes the alloantigen L^d (14) and the superantigen SEB (15), was maintained in RPMI 1640 containing 5 mM HEPES, 10% fetal bovine serum, 1.3 mM L-glutamine, 50 μ M 2-ME (RPMI medium), and 10% supernatants from Con A-stimulated rat spleen cells, as previously described (14). The 2C cells were stimulated approximately every week with mitomycin C-treated BALB/c spleen cells. Daudi, a human lymphoma that expresses class II but not class I HLA molecules, was maintained in RPMI medium. MDA-MB-453, a human breast carcinoma obtained from the American Type Culture Collection (Rockville, MD), was maintained in Leibovitz's L-15 medium containing 5 mM HEPES, 10% fetal bovine serum, 1.3 mM L-glutamine, and 50 μ M 2-ME. Hybridomas were maintained in RPMI medium.

mAbs

1B2 is a BALB/c-derived IgG1 mAb that is specific for the 2C TCR (12). 4420 is a BALB/c-derived IgG2a mAb that is specific for the hapten fluorescein (16). CT14-G4.3 is a BALB/c-derived IgG1 mAb that is specific for a 10-amino acid residue peptide derived from the c-myc protein (17). 1F4 is a hamster-derived mAb that is specific for the idiotypic region of Ab 4420 (18). BALB/c-derived Abs were purified from ascites fluid by ammonium sulfate precipitation followed by DEAE column chromatography. 1F4 was purified from culture supernatants by protein G-Sepharose chromatography. 1B2 and 1F4 Fab fragments were obtained by digestion with papain followed by G200 HPLC column chromatography (15).

Construction of single chain bispecific Ab gene

The scFv₄₄₂₀ gene, consisting of the 4420 V_L gene, a 75-bp linker gene (called 205c; 19), and the 4420 V_H gene, was amplified by PCR with a 5' primer that included a HindIII site followed by a 205c' linker gene (primer 44VL205; 5'-ATCTAACGTTGCTAGCGCAGACGATGCCAA AAAAGACGCGCGAAAAAGACGGATGCCAAAAGACGACG CCAAAAAAGATCTTGACGTCGTTATGACTCAACACCCT A-3') and a 3' primer that included a c-myc tail, two stop codons, and a HindIII site (primer 44JH-HIND III; 5'-GATTAAGCTTTGTTCTGAG GAGACGGTGACTGAGGTT-3'). The modified 4420 scFv gene was inserted into a plasmid containing the scFv_{1B2} gene (13). The scFv_{1B2} gene consists of the ompA signal sequence, the 1B2 V_L gene, a 42-bp linker gene (called 212; Ref. 19), the 1B2 V_H gene, and a 30-bp gene encoding the c-myc peptide (17). The modified 4420 scFv was inserted at the HindIII site between the 1B2 scFv V_H gene and the c-myc gene so the c-myc tail could be used for purification and detection (Fig. 1).

E. coli expression system

The scFv₂ construction was expressed using a hybrid O_t/P_R λ phage promoter in an *E. coli* strain that contains a temperature-sensitive C₁₈₅₇

repressor gene. Induction was performed as previously described (20). Briefly, small scale cultures or fermentations were grown to an A_{600} of 1.0 at 30°C, at which point recombinant protein expression was induced by temperature shift to 42°C for 1 h. Cells were pelleted, resuspended in 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM CaCl₂, and 1 mM EDTA, and disrupted by passage through a microfluidizer. After lysis, 0.1 mM PMSF was added. Unlysed cells were removed by centrifugation at 3,000 rpm and the supernatant was centrifuged at 16,000 rpm to pellet inclusion bodies.

Single chain Ab purification

Inclusion body pellets were solubilized for 4 to 12 h in 6 M guanidine hydrochloride (10 ml guanidine/cell pellet equivalent obtained from 1 liter of culture). The preparation was centrifuged at 16,000 rpm to remove insoluble material and was dialyzed against 0.1 M Tris (pH 8.0), 2 mM EDTA (pH 8.0), and 0.4 M arginine (TEA buffer) at 4°C. After dialysis, insoluble proteins were removed by centrifugation at 16,000 rpm. Fluorescein-specific scFv₂ were isolated by affinity chromatography on Sepharose 6B conjugated to 5-aminofluorescein (21). The anti-fluorescein scFv₂ Abs were eluted with 0.1 M fluorescein. Unbound fluorescein was removed by anion exchange chromatography over Dowex 1-X8. All columns were equilibrated in TEA (pH 8.0) or PBS, (pH 8.0). Fractions from columns run in TEA were dialyzed against PBS at 4°C.

Monospecific 4420 scFv Ab was purified from inclusion body pellets by fluorescein affinity chromatography, as described for scFv₂. Monospecific 1B2 scFv were purified by size exclusion of refolded protein through an HPLC Superdex G200 column. The monomeric 1B2 scFv peak was pooled and dialyzed against PBS (13).

Binding assays

To detect fluorescein binding activity, scFv₂ preparations were assayed in an indirect ELISA with FITC/BSA absorbed to wells. FITC/BSA was incubated at 10 μ g/ml in Immulon 2 wells of a 96-well plate (Dynatech, Alexandria, VA) at 4°C overnight. Wells were washed and masked with PBS containing 0.05% Tween 20, 0.1% BSA, and 100 μ g/ml thimerosal. Various concentrations of unpurified *E. coli* extract or affinity-purified preparations of scFv₂ were added to the wells. Binding was detected with the monoclonal anti-c-myc Ab, CT14-G4.3 (used as undiluted culture supernatant), followed by horseradish peroxidase-conjugated goat anti-mouse IgG (Kirkegaard and Perry) and the substrate tetramethylbenzidine. Absorbances at 450 nm were determined with a 96-well plate reader.

Fluorescein binding affinities were determined by using a competition assay with an ¹²⁵I-labeled anti-idiotypic Ab, 1F4 (18)³. Intact 4420 Ab or scFv₂ was added to Immulon 2 wells and incubated at 4°C overnight. After masking with PBS/BSA, various concentrations of fluorescein were added together with a constant amount (~200,000 cpm) of ¹²⁵I-labeled 1F4 Fab fragments. Wells were washed with PBS/BSA and the amount of radioactivity bound was monitored using a gamma counter. Fluorescein inhibition curves were prepared and the affinity of scFv₂ relative to intact Ab 4420 was determined by direct comparison of the concentration of fluorescein, which yielded 50% inhibition of 1F4 binding to intact 4420 Ab or scFv₂ Ab.

To detect scFv₂ binding to the TCR on CTL 2C, a competition assay was performed with ¹²⁵I-labeled 1B2 Fab fragments. Various concentrations of 1B2 Fab fragments or scFv₂ were added, together with a constant amount (~200,000 cpm) of ¹²⁵I-labeled 1B2 Fab fragments, to tubes containing 2 × 10⁵ 2C cells. After a 30-min incubation at room temperature, cells were washed with PBS/BSA and cell pellets were monitored in a gamma counter. Inhibition curves were prepared for unlabeled 1B2 Fab fragments and scFv₂ Abs.

Cytotoxicity assays

Target cells were incubated with 50 μ l of ⁵¹Cr (2.5 mCi/ml) for 1 h at 37°C. After washing with PBS, cells were incubated in 1 ml PBS, pH 8.5, containing 100 μ M FITC for 15 min at 37°C. Target cells were washed

³ Wilson, E., and D. M. Kranz. Binding affinities of anti-fluorescein Abs for various fluorescein-conjugates. Manuscript in preparation.

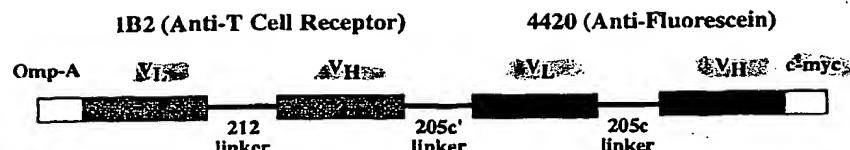


FIGURE 1. Schematic of bispecific single chain anti-TCR/anti-fluorescein Ab (scFv₂). The 1B2 V_l and V_h genes were linked by a sequence encoding the 14 amino acid 212 linker (GSTSGSGKSSEGKG) and 4420 V_l and V_h genes were linked by a sequence encoding the 25 amino acid 205c linker (SSADDAAKKDAAKKDDAKKDCG) (Ref. 19); these monospecific single chain constructions were linked by a sequence that encodes a 25 amino acid modified 205c linker, called 205c' (ASADDAAKKDAAKKD DAKKDDAKKDL). The bispecific single chain gene was inserted between the OmpA signal sequence (MKKTAIAIAVALAGF ATVAQA) and a 10 amino acid peptide from the c-myc protein (EQKLISEED).

in RPMI medium and added to round bottom wells of a 96-well plate at 2×10^3 cells/well. The 2C cells were either incubated with scFv₂ and washed with medium before addition to wells or scFv₂ was added directly to the cytotoxicity assay. For inhibition experiments, scFv₂, intact 1B2 Abs, or intact 4420 Abs were added directly to assays. For some experiments, SIgB was added directly to assays at a final concentration of 10 μ g/ml. All assays were incubated for 4 h at 37°C. Supernatants were removed and monitored with a gamma counter. Specific ⁵¹Cr release was determined as follows:

$$\% \text{ specific } ^{51}\text{Cr} \text{ release} = [(\text{experimental counts} - \text{spontaneous counts}) / (\text{maximal counts} - \text{spontaneous counts})] \times 100.$$

Results

A gene that encodes a single chain protein (scFv₂) with binding domains of both the anti-TCR Ab 1B2 and the anti-fluorescein Ab 4420 was constructed as shown in Figure 1. The 1B2 is a clonotypic Ab that reacts with the TCR from the BALB.B CTL clone 2C (12). The interantibody linker is 25 residues in length and is similar to the 205c linker described by Pantoliano et al. (19) (one residue at each end is different to accommodate restriction sites in the scFv₂ construction). A 10-residue c-myc tail was inserted at the COOH-terminus to allow detection with the anti-c-myc mAb, CT14-G4.3 (17).

Expression and purification of TCR/fluorescein bispecific Ab

A previous study from our lab showed that the single chain anti-TCR Ab 1B2 (scFv_{1B2}) was present in the insoluble fraction of the lysed *E. coli* cell pellet (13). Thus, inclusion body pellets from cells that contained the scFv₂ gene were solubilized in guanidine and dialyzed against a Tris-arginine buffer to allow refolding (22). The dialyzed sample was passed through a fluorescein column and the specific binding material was eluted with 0.1 M free fluorescein. After passage over an anion exchange resin to remove unbound fluorescein, the fluorescein-specific protein was examined by SDS-PAGE. The major protein had a 56,600-Da apparent molecular mass under reducing (Fig. 2A) and nonreducing conditions (data not shown). As expected, this was approximately twice the size of either the 1B2 scFv or 4420 scFv (31,500 Da; Fig. 2A). Approximately 1 mg of fluorescein-purified single chain was obtained from 1 L of bacterial culture. This

represents approximately 10% of the protein from inclusion bodies that migrates with an apparent molecular mass of 56,600 Da.

Recently, we have observed that monospecific 1B2 scFv can form active, noncovalently associated dimers (13). To determine whether a proportion of the bispecific Ab is also present in multimeric form, the affinity-purified sample was analyzed by HPLC under nondenaturing conditions (i.e., PBS) through a G200 column. As shown in Figure 2B, the only detectable component chromatographed, with an approximate size of 45 kDa, indicating that >90% of the affinity-purified scFv₂ is present in monomeric form. The monomeric fraction has been found to retain activity over 1 mo in storage in PBS at 4°C.

Binding properties of TCR/fluorescein bispecific Ab

It has been shown that the affinity of monovalent scFv₄₄₂₀ is approximately two- to threefold lower than that of intact 4420 Ab (23). In contrast, monovalent scFv_{1B2} has an affinity that is approximately 20-fold lower than that of intact 1B2 (13). To examine the affinity of the bispecific scFv₂ Ab for fluorescein, the amount of fluorescein required to inhibit the binding of Fab fragments derived from an anti-idiotype₄₄₂₀ Ab, 1F4, was determined (18). We have shown that this assay can accurately determine the affinity of 4420 as judged by comparison with several other fluorescein-based approaches. Radioactive iodine-labeled 1F4 Fab fragments bound to both intact 4420 and the scFv₂ with similar efficiencies (19,000 and 21,000 maximum cpm bound, respectively). This binding was inhibited by fluorescein and from the results shown in Figure 3 the affinity of scFv₂ was judged to be 2.2-fold lower than the intact Ab. Thus, the presence of another Ab-binding domain at the NH₂-terminus does not affect the ability of the anti-fluorescein domain to fold into a native conformation.

An ELISA was performed to determine whether the c-myc peptide, located at the COOH-terminus, was accessible to a monoclonal anti-c-myc Ab (17). Bispecific Ab from unpurified *E. coli* extracts or the affinity-purified

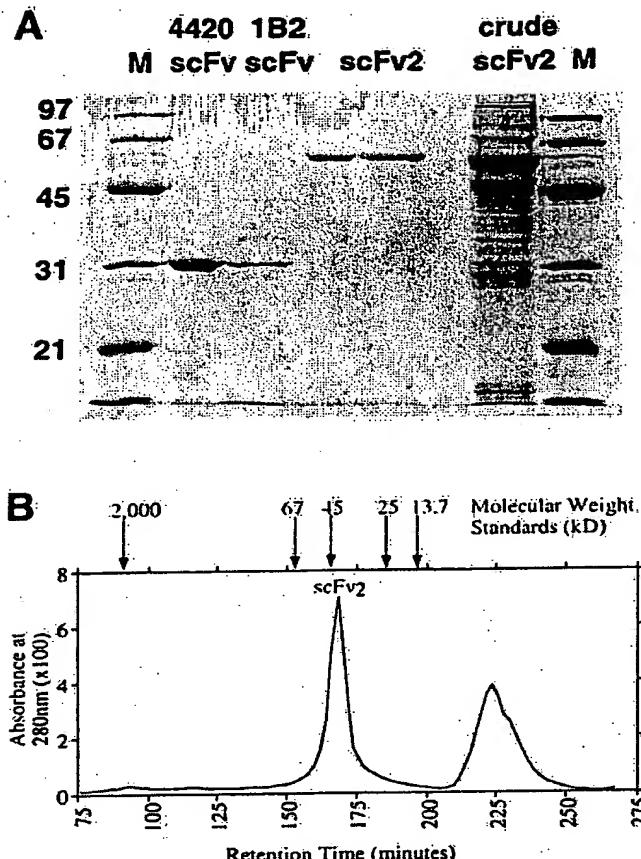


FIGURE 2. SDS-PAGE analysis and gel filtration profile of affinity purified scFv₂. *A*: SDS-PAGE of bispecific scFv₂ and monospecific 1B2 scFv and 4420 scFv. Single chain Abs were prepared as described in Materials and Methods: 1B2 scFv was purified by gel filtration chromatography and 4420scFv and scFv₂ were purified by affinity chromatography on fluorescein columns. Two preparations of scFv₂ obtained using identical protocols are shown. Crude scFv₂ represents the TEA-dialyzed preparation before affinity purification. Molecular mass markers (in kDa) are shown. Proteins were visualized by staining with Coomassie blue. *B*: Bispecific scFv₂ Ab was purified by fluorescein-Sepharose affinity chromatography in TEA, pH 8.0, and applied to a Superdex G-200 HPLC column in PBS, pH 7.3. Absorbance was monitored at 280 nm. The column was calibrated with molecular mass standards, and one peak corresponding to the monomer scFv₂ was identified. A second major component eluted at the total column volume and was probably caused by arginine from the TEA purification buffer and/or small protein degradation products.

preparations bound to FITC/BSA as detected with the anti-c-myc Ab (Fig. 4). Together, these data indicated that placement of peptide domains at both the NH₂-terminus (1B2) and COOH-terminus (c-myc) did not restrict the folding of the two V₄₄₂₀ domains.

To determine whether the fluorescein-purified bispecific Ab also contained properly refolded 1B2 domain, a com-

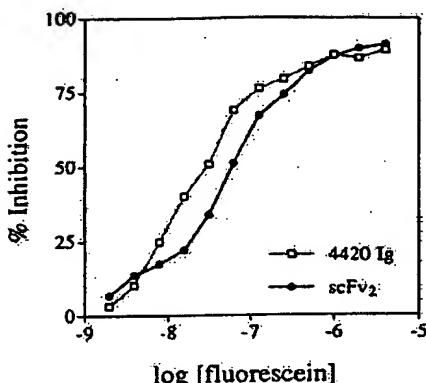


FIGURE 3. Fluorescein-binding affinity of scFv₂ Ab relative to intact 4420 Ab. Fluorescein was used to inhibit binding of scFv₂ or intact 4420 Ab by Fab fragments of the anti-idiotype mAb 1F4 (18). Various concentrations of fluorescein were added together with a constant amount (~200,000 cpm) of ¹²⁵I-1F4 Fab fragments to wells absorbed with scFv₂ or Ab 4420 at 10 μ g/ml. After 30-min incubation, wells were washed with PBS/BSA then monitored by using a gamma counter. Percentage of inhibition values were determined relative to binding in the absence of fluorescein.

petition assay was performed with ¹²⁵I-labeled Fab fragments of 1B2. As shown in Figure 5, the bispecific Ab binds to the CTL 2C, although at total protein concentrations that are 300-fold higher than unlabeled Fab fragments. There are two possible, nonmutually exclusive explanations for the observed reduction in binding by scFv₂ compared with 1B2 Fab fragments. First, the scFv₂ may indeed have a 300-fold lower affinity than 1B2 Fab fragments for the TCR on CTL 2C. Second, there may be a significant fraction of scFv₂ that has the fluorescein binding domain folded but not the 1B2 domain folded. At this time these two possibilities cannot be experimentally distinguished but it is likely that both factors contribute to the observed activity of the scFv₂. For example, if the affinity of scFv₂ for the TCR is 20-fold lower than 1B2 Fab fragments (like the scFv_{1B2}), then approximately 5% of the scFv₂ would be expected to contain both fluorescein and TCR binding domains.

Bispecific Ab activity in CTL-mediated killing assays

Because the scFv₂ has bispecific activity, it was of interest to examine whether the Ab could mediate lysis of target cells by CTL 2C. The human lymphoblastoid cell line Daudi was labeled with FITC for use as the target cell (FITC/Daudi). As expected, the L^d alloreactive CTL 2C was unable to lyse unlabeled Daudi cells, with or without the bispecific Ab (Table I, experiment 1). In contrast, FITC/Daudi were lysed when CTL 2C was either preincubated with scFv₂ or when the Ab was added directly to the CTL/target cell assay (Fig. 6). The observed lysis was

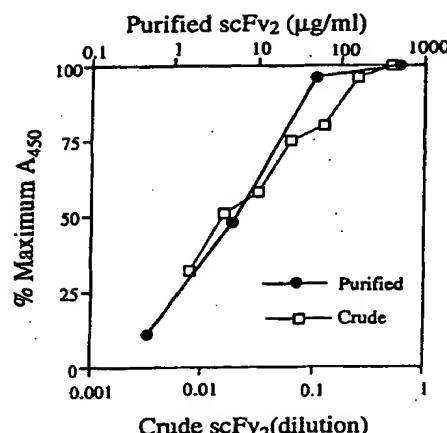


FIGURE 4. Detection of scFv₂ Ab binding to fluorescein with anti-c-myc Abs. Serial dilutions of a crude extract (inclusion bodies solubilized in guanidine and dialyzed against TEA) of *E. coli* containing the scFv₂ gene or the purified scFv₂ were added to the wells preabsorbed with FITC/BSA (10 μg/ml). After washing, Abs were detected with a monoclonal anti-c-myc Ab (CT14; 17) followed by horseradish peroxidase-labeled goat anti-mouse Ig and substrate. Data are presented as percentage of maximum A₄₅₀, which was calculated by normalizing experimental values to the absorbance at the highest concentration scFv₂ assayed. Maximum A₄₅₀ was 0.50 for crude scFv₂ and 1.1 for purified scFv₂.

dependent on the concentration of bispecific Ab. Incubation in the presence of scFv₂ was more effective than preincubation of CTL with scFv₂ before the assay, followed by washing.

The highest concentration of Ab (200 μg/ml) showed a reduced degree of killing when incubated with the cells throughout the assay (Fig. 6). This finding has been noted with chemically coupled Abs (3) and is probably a result of the inhibitory effects of binding by excess anti-fluorescein and excess anti-TCR Abs. Consistent with this possibility, monospecific 1B2 Ab or 4420 Ab each inhibit the lysis mediated by the bispecific Ab (Table I, experiment 2). The ability to mediate lysis requires the covalent linkage engineered between the two Ab domains as shown by the finding that scFv_{1B2} alone, scFv₄₄₂₀ alone, or a mixture of the two scFv were unable to mediate lysis of the target cells (Table I, experiment 3).

To confirm that other tumor cells could also be recognized and lysed in the presence of the scFv₂ Ab, the breast cancer line MDA-453 was FITC labeled and examined as a target cell (Table I, experiment 4). Although the extent of lysis was less than observed with Daudi cells, MDA-453 was also susceptible to lysis mediated by scFv₂ Ab.

Bispecific Abs have the potential to suppress T cell responses because of the inhibitory properties of the anti-TCR activity. To examine whether the concentrations that led to lysis of target cells were inhibitory, we tested the

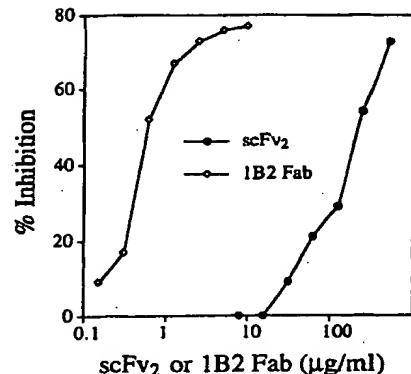


FIGURE 5. Binding of scFv₂ to the TCR on CTL clone 2C. Various concentrations of 1B2 Fab fragments or scFv₂ were added, together with a constant amount (~200,000 cpm) of ¹²⁵I-labeled 1B2 Fab fragments, to tubes containing 2 × 10⁵ 2C cells. After 30 min at room temperature, cells were washed with PBS/BSA and cell pellets were monitored in a gamma counter. Percentage of inhibition values were determined relative to binding in the absence of inhibitor. Ab concentrations are in μg/ml.

Abs in another cytolytic assay. In this case the target Daudi was incubated with the superantigen SEB, which is recognized by CTL 2C (15). This recognition is inhibited at 1B2 concentrations that are severalfold lower than inhibition of the peptide/L^d ligand recognized by 2C (13). Even at a scFv₂ concentration of 20 μg/ml, which is 100 times the level required for scFv₂ mediated lysis (as low as 200 ng/ml), recognition of SEB/Daudi was not inhibited (Table I, experiment 5).

Discussion

A simple anti-TCR/anti-fluorescein Ab was used in this study to begin engineering single chain bispecific Abs that have potentially advantageous features including, rapid purification in high yield, minimal side effects because of the deletion of C regions, and optimum tumor penetration associated with their smaller size (compared with bispecific Fab or intact Abs). The system involved two Abs that have been studied extensively for their specificities and that have been expressed as single chain monospecific Abs in *E. coli*. Use of these two Abs allowed the determination of the yield and affinity of the active bispecific Ab and the analysis of inhibitory effects that might be associated with anti-TCR activity of the bispecific Ab.

An important issue in the design of engineered bispecific Abs is the ability of each of the Ab domains to fold when expressed as a single chain with other peptides or proteins positioned at the NH₂-terminus, the COOH-terminus, or both. Purification of the 57-kDa bispecific Ab at mg/L quantities by affinity chromatography showed that the Fv₄₄₂₀ domain can fold, despite the presence of amino

Table I. Tumor cell lysis mediated by scFv₂ and CTL clone 2C

Experiment 1		% Specific ⁵¹ Cr Release	
Ab	μg/ml	FITC/Daudi	Daudi
scFv ₂	200 ^a	43	0
scFv ₂	18	24	0
Experiment 2		% Specific ⁵¹ Cr Release	
Ab	μg/ml	Inhibitor	FITC/Daudi
scFv ₂	50	1B2 Ig	58
scFv ₂	50	100 μg/ml	7
scFv ₂	50	4420 Ig	38
		100 μg/ml	
Experiment 3		% Specific ⁵¹ Cr Release	
Ab	μg/ml	FITC/Daudi	
scFv ₂	2	30	
scFv ₂	20	70	
scFv _{1B2}	2	0	
scFv ₄₄₂₀	2	0	
scFv _{1B2} + scFv ₄₄₂₀	2 + 2	0	
Experiment 4		% Specific ⁵¹ Cr Release	
Ab	μg/ml	FITC/Daudi	FITC/MDA-MB-453
scFv ₂	50	80	32
scFv ₂	50	82 ^b	41 ^b
Experiment 5		% Specific ⁵¹ Cr Release	
Ab	μg/ml	SEB/Daudi	
scFv ₂	2	89	
scFv ₂	20	92	
		87	

^a CTL 2C was preincubated with 200 μg/ml scFv₂ for 30 min then washed and added to ⁵¹Cr-labeled target cells. In all other experiments (1 to 5), Abs were added directly to the CTL/target cell assay without washing cells.

^b CTL 2C-to-target cell ratio was 15:1 in these assays. All other assays were kept at a ratio of 6:1.

acid residues that extend from the NH₂ and COOH ends. The yield was only twofold lower than the yield of single chain monospecific anti-fluorescein Abs (data not shown), suggesting that it will indeed be possible to engineer multispecific Abs using the single chain approach. Furthermore, the affinity of the anti-fluorescein domain was nearly identical with that of the monospecific single chain Ab. It remains to be determined whether the Fv₂ regions from other Abs will be sterically prevented from proper folding in this context.

The affinity and yield of the TCR binding domain were more difficult to quantitate because it was not possible to separate the issues of concentration from affinity using these approaches. From the binding studies shown in Figure 5 it is possible to conclude that the K_d of the anti-TCR domain is at least $3 \times 10^{-7} M^{-1}$ (i.e., not less than 300-fold lower than 1B2 Fab fragments; 13). If the affinity of the anti-TCR domain is the same as 1B2 scFv monomers ($\sim 2 \times 10^{-8} M^{-1}$) then the yield of the refolded Fv_{1B2} domain would be 5% of the total affinity-purified scFv₂ preparation. However, an accurate determination will require the use of TCR affinity columns, similar to the fluorescein affinity column. In fact, it is likely that the purifi-

cation and hence activity of bispecific Abs will be optimized by the use of tandem affinity columns.

A recent study showed that noncovalently associated bispecific Abs (called diabodies) could be engineered by expressing two single chain genes, each containing heterologous V_H and V_L genes from the parental Abs (11). By eliminating a linker region between heterologous V_H and V_L pairs it was suggested that the proteins would be sterically hindered from forming intrachain V_LV_H pairs and that they would consequently associate with the interchain homologous V_H and V_L preferentially. The yields in that report were also in the range of 0.3 to 1.0 mg/L of bacterial culture but the diabodies were not designed to target cells for lysis through the TCR. It should be useful to determine whether their system is superior to the single chain approach used in this report and if linker regions other than the 25-residue 205c may yield Abs that are even more efficient at mediating the lysis of target cells. Clearly, the bispecific Ab genes described in this study should allow the rapid analysis of these issues.

The results shown in Figure 6 indicated that continuous incubation of CTL and target cells with the bispecific Abs was more effective than preincubation of Ab with CTL, as

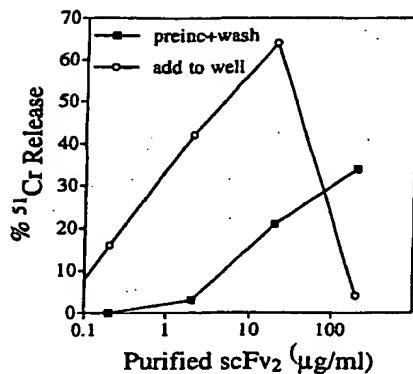


FIGURE 6. Bispecific scFv₂ Ab mediates lysis of FITC-labeled tumor cells by CTL 2C. Daudi cells were incubated with 50 µl of ⁵¹Cr (2.5 mCi/ml) for 1 h at 37°C. After washing with PBS, cells were incubated in 1 ml 100 µM FITC for 15 min at 37°C. FITC-labeled Daudi cells were washed with RPMI medium and plated at 2 × 10⁴ cells/well. The 2C cells were either preincubated with scFv₂ and washed with PBS before addition to wells (preincubated + wash) or scFv₂ was added directly to the CTL/target assay (add to well). Assays were performed at a 2C:FITC/Daudi ratio of 6:1 and incubated for 4 h at 37°C. Supernatants were removed and monitored with a gamma counter.

has been done in other studies (5). This property should be advantageous for the use of these agents *in vivo*, where it would be preferable not to have to treat patients with adoptively transferred effector cells. Finally, it was observed that the concentrations of bispecific Ab that are active in retargeting cells for lysis are considerably below the concentrations that inhibit T cell activity (Table I, experiment 5). Considerably less 1B2 Ab is required to inhibit SEB/Daudi than to inhibit recognition of the nominal Ag, p2Ca/L^d (13). Thus, it should be possible to use concentrations of bispecific Abs that are effective at mediating killing but will not inhibit normal TCR-mediated responses (24).

Acknowledgments

We thank Drs. Lisa Denzin and Edward W. Voss, Jr. for providing the hybridoma line 1F4.

References

1. Staerz, U. D., O. Kanagawa, and M. J. Bevan. 1985. Hybrid antibodies can target sites for attack by T cells. *Nature* 314: 628.
2. Perez, P., R. W. Hoffman, S. Shaw, J. A. Bluestone, and D. M. Segal. 1985. Specific targeting of cytotoxic T cells by anti-T3 linked to anti-target cell antibody. *Nature* 316:354.
3. Liu, M. A., D. M. Kranz, J. T. Kurnick, L. A. Boyle, R. Levy, and H. N. Eisen. 1985. Heteroantibody duplexes target cells for lysis by cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* 82:8648.
4. Fanger, M. W., P. M. Morganelli, and P. M. Guyre. 1992. Bispecific antibodies. *Cr. Rev. Immunol.* 12:101.
5. Titus, J. A., M. A. Garrido, T. T. Hecht, D. F. Winkler, J. R. Wunderlich, and D. M. Segal. 1987. Human T cells targeted with anti-T3 cross-linked to antitumor antibody prevent tumor growth in nude mice. *J. Immunol.* 138:4018.
6. Nitta, T., K. Sato, H. Yagita, K. Okumura, and S. Ishii. 1990. Preliminary trial of specific targeting therapy against malignant glioma. *Lancet* 335:368.
7. Bird, R. E., K. D. Hardman, J. W. Jacobson, S. Johnson, B. M. Kaufman, S.-M. Lee, T. Lee, S. H. Pope, G. S. Riordan, and M. Whitlow. 1988. Single chain antigen-binding proteins. *Science* 242:423.
8. Huston, J. S., D. Levinson, M. Mudgett-Hunter, M.-S. Tai, J. Novotny, M. N. Margolies, R. J. Ridge, R. E. Brucolieri, E. Haber, R. Crea, and H. Opermann. 1988. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 85:5879.
9. Wright, A., S.-U. Shin, and S. L. Morrison. 1992. Genetically engineered antibodies: progress and prospects. *Cr. Rev. Immunol.* 12: 125.
10. Pluckthun, A. 1992. Mono- and bivalent antibody fragments produced in *Escherichia coli*: engineering, folding and antigen binding. *Immunol. Rev.* 130: 151.
11. Holliger, P., T. Prospero, and G. Winter. 1993. "Diabodies": small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. USA* 90:6444.
12. Kranz, D. M., S. Tonegawa, and H. N. Eisen. 1984. Attachment of an anti-receptor antibody to non-target cells renders them susceptible to lysis by a clone of cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* 81:7922.
13. Schodin, B. A., and D. M. Kranz. 1993. Binding affinity and inhibitory properties of a single-chain anti-T cell receptor antibody. *J. Biol. Chem.* 268:25722.
14. Kranz, D. M., D. H. Sherman, M. V. Sitkovsky, M. S. Pasternack, and H. N. Eisen. 1984. Immunoprecipitation of cell surface structure of cloned cytotoxic T lymphocytes by clone-specific antisera. *Proc. Natl. Acad. Sci. USA* 81:573.
15. Soo Hoo, W. F., and D. M. Kranz. 1993. Role of CD8 in staphylococcal enterotoxin B-mediated lysis by cytotoxic T lymphocytes. *J. Immunol.* 150:4331.
16. Kranz, D. M., and E. W. Voss, Jr. 1981. Partial elucidation of an antihapten repertoire in BALB/c mice: comparative characterization of several monoclonal anti-fluoresceyl antibodies. *Mol. Immunol.* 18: 889.
17. Evan, G. I., G. K. Lewis, G. Ramsay, and J. M. Bishop. 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* 5:3610.
18. Weidner, K. M., L. K. Denzin, M. L. Kim, W. D. Mallender, S. D. Miklasz, and E. W. Voss, Jr. 1993. Elicitation of distinct populations of monoclonal antibodies specific for the variable domains of monoclonal anti-fluorescein antibody 4-4-20. *Mol. Immunol.* 30:1003.
19. Pantoliano, M. W., R. E. Bird, S. Johnson, E. D. Asel, S. W. Dodd, J. F. Wood, and K. D. Hardman. 1991. Conformational stability, folding, and ligand-binding affinity of single chain Fv immunoglobulin fragments expressed in *Escherichia coli*. *Biochemistry* 30:10117.
20. Soo Hoo, W. F., M. J. Lacy, L. K. Denzin, E. W. Voss, Jr., K. D. Hardman, and D. M. Kranz. 1992. Characterization of a single chain T cell receptor expressed in *E. coli*. *Proc. Natl. Acad. Sci. USA* 89: 4759.
21. Kranz, D. M., J. N. Herron, and E. W. Voss, Jr. 1982. Mechanisms of ligand binding by monoclonal anti-fluoresceyl antibodies. *J. Biol. Chem.* 257:6987.
22. Buchner, J., and R. Rudolph. 1991. Renaturation, purification, and characterization of recombinant Fab fragments produced in *Escherichia coli*. *Biotechnology* 9:157.
23. Bedzyk, W. D., K. M. Weidner, L. K. Denzin, L. S. Johnson, K. D. Hardman, M. W. Pantoliano, E. D. Asel, and E. W. Voss, Jr. 1990. Immunological and structural characterization of a high affinity anti-fluorescein single chain antibody. *J. Biol. Chem.* 265:18615.
24. Waldmann, T. A. 1992. Immune receptors: targets for therapy of leukemia/lymphoma, autoimmune diseases and for prevention of allograft rejection. *Annu. Rev. Immunol.* 10:675.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.